CYTOKINES AND GRAFT FUNCTION IN HEART TRANSPLANT RECIPIENTS

cytokines en transplantaatfunctie na harttransplantatie

ISBN 90-9016400-6
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Cytokines and graft function in heart transplant recipients

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Omslag: 'Erotic Sunset', geschilderd door mijn moeder Nancy Kruseman (overleden op 14 januari 2000)

Drukwerk: Print Partners Ipskamp, Enschede

CYTOKINES AND GRAFT FUNCTION IN HEART TRANSPLANT RECIPIENTS

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Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
Rector Magnificus

Prof.dr.ir. J.H. van Bemmel

en volgens besluit van het College voor Promoties.
De openbare verdediging zal plaatsvinden op

woensdag 4 december 2002 om 13.45 uur
door

Hester Antoinette de Groot-Kruseman
geboren te De Bilt

Promotiecommissie

Promotor:	Prof.dr. W. Weimar
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Financial support by the Netherlands Heart Foundation for the publication of this thesis is gratefully acknowledged

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CHAPTER 1

GENERAL INTRODUCTION

Heart transplantation

History of clinical heart transplantation

After the first experimental attempts to carry out heart transplantation in dogs at the beginning of the 20th century,[1] the first successful human heart transplantation was performed in 1967 by Christiaan Barnard in Cape Town, South-Africa.[2] Further technical improvements and the development of the bioptome for the diagnosis of acute rejection by histological grading of endomyocardial biopsies in 1973, followed by the introduction of the immunosuppressive drug cyclosporine A in 1982, initiated the acceptance of heart transplantation as treatment for end-stage heart failure.[3,4] Up till now, more than 57.000 heart transplantations have been performed in more than 200 hospitals worldwide.[5] In the Netherlands the first heart transplantation was carried out in 1984 in Rotterdam,[6] where to date more than 400 heart transplantations have been performed.

Complications after heart transplantation

Currently, the overall 1- and 5-year survival rates after heart transplantation are 82% and 68% respectively.[5] During the last decade, survival rates have not improved further because more critical ill patients have been transplanted and donor acceptance criteria have been adjusted to increase the number of available donor hearts.[7] Causes of death early after transplantation include primary graft failure and acute rejection, while mortality after the first year is mostly due to malignancies and graft vascular disease.[5]

The most important complication after heart transplantation is rejection, which is the result of the immune reaction of the recipient against the non-self donor heart, which leads to allograft damage.[7] To prevent rejection, transplant recipients are treated with immunosuppressive agents during the rest of their lives. These immunosuppressants, however, may cause complications such as infection and malignancy next to specific side effects such as nephropathy (by cyclosporine and tacrolimus), or gastrointestinal problems (by mycophenolate mofetil).[7]

Rejection after heart transplantation

Hyperacute rejection

Hyperacute rejection refers to a very rapidly occurring inflammatory reaction (within minutes to hours after transplantation) mediated by preformed circulating anti-donor

antibodies which bind to the vascular endothelium of the graft.[8,9] This results in complement activation and leads to vascular thrombosis ending in rapid necrosis and destruction of the donor heart. Preformed circulating antibodies may originate from blood-group incompatibility, blood transfusions, pregnancies or previous transplants. To avoid this type of rejection, serum from the transplant candidate is screened for the presence of panel-reactive antibodies before transplantation.[10]

Acute cellular rejection

Acute rejection refers to a T cell mediated inflammatory response within the allograft. This usually occurs within weeks to months after transplantation.[9] Acute rejection is initiated by the presentation of foreign allo-antigens to the T cell receptor (TCR) by antigen-presenting cells (APC's) such as B cells, macrophages and dendritic cells. Optimal T cell activation requires not only the interaction between the TCR and the foreign allo-antigen, but also interactions between costimulatory molecules on APC's and their ligands on T cells.[11] Antigen stimulation in conjunction with costimulation drives the production of cytokines resulting in proliferation and differentiation of helper T cells and donor specific cytotoxic T cells leading to the clinical signs of acute rejection.[12-14]

Because acute cellular rejection after heart transplantation is clinically asymptomatic at an early stage, the diagnosis is based on the histological examination of endomyocardial biopsies (EMB) obtained from the right side of the interventricular septum.[3] In Rotterdam such biopsies are performed weekly during the first 6 weeks, biweekly up to 3 months, monthly up to 6 months and bimonthly up to the first year. In 1990, cardiac pathologists united in the International Society of Heart and Lung Transplantation (ISHLT) formulated the ISHLT scale for grading of cardiac rejection.[15] The rejection grade is more severe with increasing intragraft accumulation of lymphocytes and increasing evidence of myocyte damage. Grade 0: no rejection, grade 1A: focal infiltrate without necrosis, grade 1B: diffuse but sparse infiltrate without necrosis, grade 2: one focus with aggressive infiltration and/or focal myocyte damage, grade 3A: multifocal aggressive infiltrates and/or myocyte damage, grade 3B: diffuse inflammatory process with myocyte necrosis, grade 4: diffuse aggressive polymorphous infiltrate with myocyte necrosis, usually with hemorrhage and vasculitis. Treatment of acute cellular rejection is considered necessary from grade 3A. Treatment consists of high doses of methylprednisolone or, in case of ongoing or frequently recurring rejection, with anti-T cell antibodies (ATG or OKT3).

Acute humoral (vascular) rejection

Humoral (vascular) rejection refers to a specific type of acute rejection in which the graft vasculature is primary target for the immunological response mediated by donor-specific antibodies. This type of rejection usually occurs in the first month after transplantation.[16] Acute humoral rejection has a worse prognosis than acute cellular rejection and has been associated with severe left ventricular dysfunction and decreased survival rates.[16-19] Light microscopic findings of diffuse endothelial cell swelling and interstitial edema are often subtle and nonspecific. Therefore, the diagnosis of humoral rejection is based on evidence of vascular immunoglobulin complexes and complement deposition as detected by immunofluorescence.[16,17] Treatment strategies include plasmapheresis, photopheresis, corticosteroids, or cyclophosphamide administration.[9,17]

Graft vascular disease (chronic rejection)

Chronic rejection after heart transplantation is named graft vascular disease (GVD) and refers to an accelerated form of atherosclerosis of the coronary arteries of the graft resulting in concentric intimal thickening.[20,21] It usually develops slowly over a period of months or years, affecting ultimately almost all heart transplant recipients. GVD is a diffuse process that starts in small distal vessels and finally involves all intramyocardial and epicardial arteries of the allograft. Pathologic examination reveals concentric intimal hyperplasia and accumulation of smooth muscle cells, macrophages, and T cells.[21] Despite extensive intimal proliferation, the media is rarely thickened and sometimes becomes even narrower than under normal conditions due to compensatory mechanisms.[22]

Although the pathogenesis of GVD is not completely understood, it is likely that its development is a complicated interplay between allo-antigen dependent and independent factors resulting in repetitive vascular injury and a sustained inflammatory response.[23,24] The cascade of events presumably starts with endothelial damage within the allograft, triggered by brain death, organ preservation, mechanical trauma, ischemia, and reperfusion. Subsequent repetitive damage to the endothelial barrier due to the immunologic response (acute rejection episodes, infections) results in a response-to-injury mechanism leading to upregulated expression of cytokines and adhesion molecules.[25,26] The attracted network of macrophages, T cells, endothelial cells and smooth muscle cells in turn generate cytokines that promote extracellular matrix formation and the replication and migration of vascular smooth muscle cells leading to remodeling and occlusion of the arteries.[23,24,27]

The clinical presentation of GVD is either silent myocardial infarction (without angina pectoris due to denervation of the graft) with loss of graft function, or sudden death.[22]

The incidence of GVD can be monitored by coronary angiography (at our center GVD than is defined as the presence of any vascular wall irregularity including minimal wall changes) or by the more sensitive intravascular ultrasound (IVUS) examination. Currently, no therapy for GVD is available and retransplantation is the only definitive treatment. In view of the current organ shortage, however, retransplantation is a controversial option.[20,22]

The role of cytokines

Cytokines in general

Cytokines are low-molecular weight mediators of the immune response that include interleukins, interferons, chemokines, and growth factors.[28] Cytokines are responsible for the intercellular communication between cells involved in the immune response. Most cytokines can be made by a broad range of cell types including parenchym cells such as endothelial cells, smooth muscle cells, and cardiomyocytes, and inflammatory cells such as T cells, B cells, NK cells, and macrophages. Cytokines are rarely produced individually, but rather operate in a network of many other cytokines and immune mediators.[29] The production of cytokines is inducible and after synthesis they are rapidly secreted and they have a short half-life. Cytokines are active in low concentrations and their activity is mostly local on the cell of origin (autocrine) or on neighbour cells (paracrine). Characteristic for cytokines is that they can influence different cell types (pleiotropism), and that different cytokines can have the same function (redundancy). The action of cytokines is mediated through specific cell surface receptors that transmit the cytokine-receptor interaction into an intracellular signal. In most cases cytokine production triggers upregulation of their own cytokine receptor on target cells.[28]

Cytokine cascade after transplantation

After transplantation cytokines regulate the allo-antigen dependent and independent inflammatory response.[30] Graft endothelial cells produce proinflammatory cytokines (interleukin-1 (IL-1), interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α)) upon exposure to various environmental stimuli (such as brain death, ischemia and reperfusion).[24,30] These cytokines enhance allograft reactivity by increasing major histocompatibility (MHC) antigen and adhesion molecule expression. Furthermore, they stimulate endothelial cells to produce additional cytokines such as IL-1, IL-6, IL-8, monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α), IFN-γ inducible protein-10 (IP-10), basic fibroblast growth factor (bFGF), platelet derived growth factor (PDGF-A), and transforming growth factor-β (TGF-β).[26] The chemokines IL-8, MCP-1, MIP-1α, and IP-10 attract circulating host leukocytes towards the

inflammatory tissue site.[31,32] IL-1 and IL-6 are able to promote the adhesion and activation of the attracted leukocytes, leading to another wave of cytokine production (monocytes produce IL-1, IL-6, IL-8, and MCP-1, and T cells produce IFN-γ, TNF-α, and IL-2) that stimulates migration of these cells into the allograft.[25,33] The growth factors bFGF, PDGF-A, and TGF-β on the other hand stimulate tissue repair of the damaged endothelial cells.[25] Once recruited into the allograft numerous additional cytokines are produced by activated macrophages (IL-15, MCP-1, TGF-β) and T cells (IL-2, IFN-γ, TNF-α).[28,34] IL-15 and IL-2 are able to stimulate proliferation and differentiation of T cells,[30,35] while MCP-1, TGF-β, and TNF-α enhance macrophage function.[28,30] This process provides the basis for subsequent T cell mediated immune responses leading to rejection.

Cytokine measurements in heart transplant recipients

Transplantation is associated with an inflammatory response and increased concentrations of cytokines in the heart and the blood. Serum and plasma levels of various cytokines have been correlated to the presence of histological evidence of acute cellular rejection. Although one study reported higher serum levels of IL-2, IL-6, IL-8, and TNF-α during acute rejection,[36] others could not confirm these results.[37-40] For immunologic studies intragraft measurements are preferable because cytokines are produced and active in a local manner and intragraft measurements of cytokine expression often differ from the measurements in blood.[38,39] Because cytokines operate at low concentrations, the amount of most cytokines within the graft is low and quantitative evaluation of intragraft cytokines at the protein level is for most of the cytokines difficult. In contrast, messenger RNA (mRNA) expression levels reflect gene activation at an early timepoint and can be measured after amplification of the signal by polymerase chain reaction (PCR). Cytokine mRNA expression levels did show a good correlation with cytokine protein expression in situ.[41] Therefore, several studies measured intragraft cytokine expression levels in relation to acute and chronic rejection after clinical heart transplantation.[42] Intragraft cytokine mRNA expression that has been reported to correlate with acute rejection episodes include IL-1, IL-2, IL-6, IL-8, IFN-γ, TNF-α, and PDGF.[39,41,43-47] While intragraft mRNA expression of various growth factors (PDGF, TGF-β, bFGF, acidic fibroblast growth factor (aFGF)) has been reported to correlate with graft vascular disease.[48-52] Moreover, measurements of cytokine protein production by cultured graft infiltrating cells from cardiac transplant recipients revealed an association between IL-2 and IFN-γ production and the development of acute and chronic rejection.[34,53]

Heart allograft function

Left ventricular diastolic function of the normal heart

The diastolic phase of the heart starts with isovolumic relaxation, when the left ventricular pressure falls without a change in volume. The mitral valve opens when the pressure in the relaxing left ventricle decreases below the left atrial pressure. The major part of left ventricular filling occurs immediately on opening of the mitral valve when the blood from the left chamber is abruptly released into the left ventricle. This rapid filling phase is followed by a phase of slow filling (diastasis) with a gradual rise in atrial and ventricular pressure and ventricular volume. Finally, ventricular filling is completed by an additional transfer of blood from the atrium to the ventricle as a result of atrial contraction. In this normal situation, the contribution of atrial contraction to filling of the ventricle is small.

Graft function after heart transplantation

Donor heart diastolic function is often abnormal in the early days after the transplant procedure, as a result of reduced ventricular distensibility and residual volume overload. The active relaxation proces is hampered by the disappearance of noradrenalin activation (denervation of the donor heart) and by longstanding ischemia. Passive relaxation may be reduced by pericardial effusion or by mechanical interference of the cardiac chambers especially in case of right ventricular dilatation as a consequence of pulmonary hypertension.[54,55] This diastolic dysfunction results in a restrictive filling pattern characterized by high atrial and high diastolic ventricular pressures leading to early mitral valve opening (shortening of the isovolumetric relaxation period) and an increased early inflow velocity. Because of the elevated ventricular diastolic pressure, atrial contraction results in a lower flow velocity during the final part of the filling process. Usually, this pattern will normalize during the first postoperative weeks as ischemia subsides and the volume status has returned to normal.[56] Significant diastolic dysfunction early after transplantation as measured by echocardiography has been associated with reduced patient survival.[57]

Echocardiographic indices of acute cellular rejection

Acute cellular rejection is characterized by mononuclear cell infiltration and interstitial edema resulting in increased cardiac mass, and by cardiomyocyte necrosis. This may lead to increased myocardial stiffness and abnormal relaxation.[57-59] The hemodynamics in rejecting patients resemble the pattern early after transplantation and is characterized by rapid increase in left ventricular diastolic pressure during rapid filling and a high left atrial pressure. Under these circumstances most of the filling occurs during the rapid

early filling phase with increased early diastolic flow velocity (E), decreased atrial flow velocity (A), and shortened deceleration time (DET), representing a restrictive filling pattern. Furthermore, increased pressure in the left atrium causes early opening of the mitral valve with a shortened isovolumetric relaxation period (IRP = time interval from the aortic valve closure signal to the onset of forward flow in diastole). Moreover, the pressure half time (PHT = time required for the maximum transmitral pressure gradient during diastole to fall by half) tends to decrease because of the rapid pressure increase in the left ventricle in the diastolic phase.

Because increased cardiac mass, myocardial stiffnes and abnormal relaxation can be measured non-invasively by echo-dopplercardiography, several investigators have proposed the use of this technique instead of EMB for early diagnosis of acute rejection.[59,60] Indeed, before the introduction of cyclosporine various diastolic function parameters as measured by echocardiography have been reported to change rapidly during acute rejection. After the introduction of cyclosporine, however, the course of acute rejection became more gradual, with less pronounced morphologic and functional changes and loss of sensitivity of echocardiographic indices.[61,62] Hence, many authors did not find significant changes in diastolic filling parameters in individual patients during different rejection grades.[63-66] Although in a few cases a progressive shortening of IRP and PHT and increased E with increasing acute rejection severity has been reported, no single parameter was sensitive enough to be clinically useful in the individual patient.[58,62,67]

Cytokines and graft function

The lack of association between histological rejection grades and hemodynamic parameters and the observations that diastolic dysfunction can precede histological signs of rejection,[56] or can reflect histologically undiagnosed humoral (vascular) rejection,[68] leads to the hypothesis that graft dysfunction after heart transplantation might be attributable to the local release of cytokines independent of cellular infiltrates.[69,70] The local production of cytokines by small numbers of T cells and macrophages (not enough to represent histological rejection) may lead to changes in graft dimensions and/or graft function.[71] Indeed, even at lower levels of acute rejection, cardiac allografts contain persistently abnormal levels of cytokines.[72] This upregulated cytokine expression may trigger capillary leakage resulting in edema and depression of myocyte function.[73,74] Potential mechanisms by which cytokines influence heart function are stimulation of the nitric oxide system, inhibition of the β-adrenoceptor system, and intervention in the intracellular calcium homeostasis.[73] Cytokines directly implicated to play a role in cardiac dysfunction include TNF-α, IL-1, IL-2, IL-6, and IFN-γ.[69] A few studies

investigated the role of cytokines in the pathophysiology of cardiac allograft dysfunction. Elevated systemic release of IL-6, and TNF-α correlated with diastolic and systolic allograft dysfunction and increased wall thickness independent of rejection.[73,75] Furthermore, intragraft expression of IL-6, IL-10, TNF-α and TGF-β mRNA was associated with allograft dysfunction.[71]

Apoptosis

Apoptosis after heart transplantation

Apoptosis is a form of cell death characterized by shrinkage of the cell, DNA fragmentation, and the formation of membrane-bound apoptotic bodies followed by rapid fagocytosis of the apoptotic bodies by neighbouring cells. In contrast to necrosis, apoptosis prevents leakage of intracellular contents that induce an inflammatory response. Under physiological conditions the occurence of apoptosis in tissues is rare.[76] After heart transplantation, however, apoptosis might occur both in cardiomyocytes as effector mechanism of tissue damage, and in T cells to downregulate the allo-immune response.[77] Cardiomyocyte apoptosis representing myocardial damage after transplantation has been demonstrated in a few studies.[77-79] However, apoptosis of cardiomyocytes has been observed in all degrees of rejection. Thus, the presence of apoptotic cardiomyocytes is not indicative for rejection severity. Besides the apoptosis of cardiomyocytes, apoptosis of graft infiltrating T cells has been observed in heart allografts.[78,80,81] This suggests that apoptosis also can play a favorable immunoregulatory role by decreasing the amount of alloreactive T cells.

T cell apoptosis

Apoptosis of T cells is of great importance for the homeostasis of the immune system. It prevents extensive tissue damage or autoimmunity by limiting the size and scope of the immune response.[77,82] There are two distinct mechanisms of T cell apoptosis. The first is passive cell death that occurs when activated T cells are deprived of growth factors (cytokine withdrawal). Passive cell death is induced through increased mitochondrial permeability and cytochrome C release, which activates downstream caspases. The second pathway of T cell apoptosis is activation induced cell death (AICD), which occurs in repetitively stimulated T cells. AICD is largely mediated through interaction of the cell surface Fas molecule (CD95) with its corresponding ligand FasL. This triggers binding of procaspase-8 to the intracellular death domain of the receptor followed by activation of caspase-8. Caspase-8 subsequently triggers the activation of a cascade of caspases that are responsible for the proteolytic cleavage of various proteins resulting in the morphologic appearance of apoptosis.[77] Induction of AICD occurs preferentially at

high antigen concentrations. Moreover, the T cell growth factor IL-2 has been implicated in priming mature T cells for AICD.[77,83] For example, treatment with anti-CD25 mAb downregulates both IL-2 and FasL and may therefore inhibit AICD of graft intiltrating T cells.[84] Apoptotic cell death is tightly controlled by a collection of proteins that inhibit apoptosis. A specific inhibitor of AICD triggered by Fas-FasL interaction is FLICE inhibitory protein (FLIP).[85] T cell apoptosis is closely related to immune tolerance because tolerance to a transplant might be induced if donor-reactive cells can be efficiently deleted by apoptosis.[77,83,86]

Aim of this thesis

General aim

Allo-antigen dependent and independent factors early after heart transplantation lead to a response-to-injury mechanism that may determine later outcome in terms of acute rejection, chronic rejection, graft dysfunction, and mortality.[13,24,30,53] The aim of this thesis is to elucidate the role of cytokines in the cascade of events that lead to acute rejection, chronic rejection and graft dysfunction after heart transplantation. We hypothesize that intragraft cytokine mRNA expression patterns are related to acute cellular rejection, early signs of chronic rejection, and to changes in graft dimensions and function. In addition, the role of ischemia, immunosuppressive therapy, and apoptosis in mediating the immune response is studied.

Objective of the studies

In **Chapter 2** the intragraft cytokine mRNA expression levels of the chemokine MCP-1 and the growth factor bFGF in the immediate post-operative phase is evaluated by analysis of endomyocardial biopsies sampled at the time of transplantation and biopsies sampled in the first week posttransplant. Messenger RNA expression levels of MCP-1 and bFGF are studied in relation to ischemia, immunosuppression, and the development of rejection. In **Chapter 3** the role of intragraft expression of TGF-β and PDGF-A in the development of graft vascular disease is studied (**3a**), as well as the effect of the immunosuppressive drug cyclosporine A on the expression levels of these growth factors (**3b**). In **Chapter 4** these findings are extended and changes in cytokine profile in time and during acute cellular rejection are studied during the first three post-operative months. For this study we investigated a broad range of cytokines involved in the response-to-injury process after heart transplantation (TNF-α, MCP-1, TGF-β, PDGF-A, and bFGF).

In **Chapter 5** heart function parameters as measured by echo-dopplercardiography during the first three post-operative months are studied in time and in relation to acute

rejection. Subsequently, the cytokine profiles discussed in Chapter 4 are analyzed in relation to these echo-dopplercardiographic measurements. In **Chapter 6** the effect of local interleukin-2 as expressed during acute cellular rejection on graft dimensions and heart function as measured by echo-dopplercardiography is described. In **Chapter 7** the role of interleukin-2, FasL and FLIP in apoptosis of infiltrated alloreactive T cells is investigated in relation to histological signs of acute rejection.

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CHAPTER 2

FAILURE TO DOWN-REGULATE INTRAGRAFT CYTOKINE mRNA EXPRESSION SHORTLY AFTER CLINICAL HEART TRANSPLANTATION IS ASSOCIATED WITH HIGH INCIDENCE OF ACUTE REJECTION

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Journal of Heart and Lung Transplantation 2001; 20 (5): 503 - 510.

Abstract

Brain-death, ischemia and reperfusion damage have been implicated as initial factors that lead to a cascade of immunologic events that result in allograft rejection in experimental animals. Cytokines are thought to play a central role in this process. Therefore, we evaluated intragraft cytokine mRNA expression at an early stage after clinical heart transplantation and related these data to ischemia, immunosuppression, and rejection. We sampled endomyocardial biopsies at 30 minutes (EMB 0) and at 1 week (EMB 1) after transplantation from 20 cardiac allograft recipients. Intragraft monocyte chemoattractant protein (MCP-1) and basic fibroblast growth factor (bFGF) mRNA expression levels were quantitatively measured using competitive template RT-PCR. We measured significantly lower MCP-1 and bFGF mRNA expression levels in EMB 1 compared with EMB 0 (MCP-1, $p = 0.006$; bFGF, $p = 0.019$). We found no direct correlation between the cytokine mRNA expression levels in EMB 0 or EMB 1 and ischemic times, induction therapy, or cyclosporine whole blood trough levels. Patients with a high incidence of acute rejection episodes (>2 in the first year) had higher bFGF mRNA expression levels ($p = 0.009$) and comparable MCP-1 mRNA expression levels ($p = 0.378$) at 1 week, compared with patients with a lower rejection incidence. The MCP-1 and bFGF mRNA expression levels in the first week were not associated with the development of graft vascular disease in the first year post-transplant. We found a significant decrease of intragraft MCP-1 and bFGF mRNA expression levels in the first post-operative week. Patients with a high incidence of acute rejection had higher bFGF mRNA expression levels in their first week biopsy. Therefore, we conclude that patients who fail to down-regulate their bFGF mRNA expression early after transplantation are at higher risk for acute rejection.

Introduction

Up-regulation of intragraft cytokine production early after transplantation because of surgery, ischemia, and reperfusion injury may be the initial factor that leads to clinical complications such as acute or chronic rejection. According to the response-to-injury theory, damaged endothelium within the graft triggers an inflammatory response that includes the secretion of cytokines. These cytokines (for example interleukin (IL)-1, IL-6, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1, platelet derived growth factor (PDGF) and fibroblast growth factor (FGF)) play a role in the migration, activation, and proliferation of monocytes and T-lymphocytes, which leads to allograft rejection.[1,2,3] Two cytokines potentially involved in this early cascade are MCP-1 and basic FGF (bFGF).[4,5,6]

Monocyte chemoattractant protein-1 is a chemoattractive cytokine for monocytes, T-lymphocytes and natural killer cells, and can be released by endothelial cells, monocytes, fibroblasts, and T-lymphocytes.[6,7] After exposure to ischemia and reperfusion injury, MCP-1 messenger RNA (mRNA) and protein production in canine myocardium rapidly up-regulated, preceding the influx of leukocytes and monocytes into the heart tissue.[8,9] Russell et al.[5] demonstrated that MCP-1 mRNA expression significantly increased in rat cardiac allografts at day 7 post-transplant compared with native hearts. This increase was associated with intimal thickening in coronary arteries, characteristic for chronic rejection. In experimental skin transplantation, MCP-1 gene expression up-regulated at days 3 to 9 post-transplant, which correlated with mononuclear cell infiltration (at day 3) and acute rejection.[10] Thus, experimental studies have shown that various allogeneic events and non-allogeneic factors can trigger the release of MCP-1 leading to cellular infiltration and acute or chronic rejection.

The growth factor bFGF (= FGF-2) is mainly produced by damaged/activated endothelial cells and macrophages and is involved in angiogenesis, tissue repair, and T-cell activation.[11-13] Non-allogeneic events such as ischemia and reperfusion have been shown to increase bFGF protein expression in myocardium.[14] In vitro FGF can trigger activation of a sub-set of CD4+ T cells, which may affect the development of allograft rejection.[12,15] In vivo, bFGF mRNA expression increased at day 5 in rat hind-limb allografts, which correlated with time of acute rejection.[16] Furthermore, early FGF expression has been associated with development of graft vascular disease at 2 years after clinical heart transplantation.[4] These studies indicate that expression of FGF early after transplantation might play a role in the development of acute and chronic allograft rejection.

The aim of the present study was to evaluate intragraft cytokine mRNA expression in the immediate post-operative phase after clinical heart transplantation in relation to ischemia, immunosuppression, and development of rejection. Therefore, we analysed expression levels of MCP-1 and bFGF mRNA in endomyocardial biopsies (EMB) from 20 heart transplant recipients, sampled at 30 minutes and 1 week post-transplant, and related these data to the duration of ischemia, immunosuppressive therapy, and the occurrence of acute rejection and graft vascular disease.

Patients and methods

Patients

We studied 20 cardiac allograft recipients transplanted between January 1995 and September 1998. Thirteen patients received immunosuppressive induction therapy with horse anti-thymocyte globulin (ATG). The remaining 7 patients received no induction

therapy. Maintenance immunosuppressive therapy consisted of a combination of cyclosporine (CsA) and low-dose steroids. We monitored CsA trough levels in whole EDTA blood using enzyme immunoassay (EMIT, Behring Diagnostics, Cupertino, CA, USA). We monitored for rejection using EMB and graded rejection according to the guidelines of the International Society for Heart and Lung Transplantation (ISHLT).[17] None of the patients suffered from acute rejection, defined as ISHLT grade ≥ 3A, during the first 2 weeks post-transplant. To determine expression levels of MCP-1 and bFGF, we studied 2 EMBs from each patient, 1 taken at 30 minutes (EMB 0) and 1 taken after the first week post-transplant (EMB 1).

RT-PCR

Total RNA was extracted from snap-frozen EMB and subsequently cDNA was synthesized with random primers as described previously in detail.[18] Aliquots of cDNA (representing 1/20 EMB) were directly used for PCR amplification, using sequence-specific primers for MCP-1 (sense: 5'-TAG-CAG-CCA-CCT-TCA-TTC-C-3', anti-sense: 5'-TTC-CCC-AAG-TCT-CTG-TAT-CT-3') and bFGF (sense: 5'-GGC-TTC-TTC-CTG-CGC-ATC-CA-3', anti-sense: 5'-GCT-CTT-AGC-AGA-CAT-TGG-AAG-A-3'). To estimate the relative initial amount of functional MCP-1 and bFGF mRNA in EMB, a competitive template RT-PCR assay was used and a comparison was made against the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

To obtain a standard curve for MCP-1, bFGF, and GAPDH, known amounts of internal control fragment were added, in different dilutions, to constant amounts of sample cDNA for competitive co-amplification. We designed the internal control to generate a smaller PCR product to allow differentiation between the amplified target and the internal control. Sample cDNA and internal control, 5 μl each, were added to a 90-μl PCR mixture containing 10 mM Tris-HCl, pH 8.3; 50 mM KCl; 1.5 mM $MgCl_2$; 0.2 mM of each dATP, dCTP, dTTP, dGTP; and 2 U Ampli Taq Gold (PE Biosystems, Norwalk CT, USA) and overlaid with 100 μl mineral oil (Sigma, St Louis MO, USA) before PCR reaction in a DNA thermal cycler model 480 (PE Biosystems) under the following conditions. After a 10-minute 94°C denaturation step, samples underwent 40 cycles of 1-minute denaturation at 94°C; 2-minute annealing at optimal temperatures for GAPDH (60°C), MCP-1 (56°C) or bFGF (58°C); and 3-minute extension at 72°C. The last cycle was extended 7 minutes at 72°C.

Positive control samples were produced by messenger RNA extraction and cDNA synthesis from 10^6 human spleen cells stimulated with 1% phytohemagglutin-M (Difco, Detroit MI, USA) for 24 hours at 37°C. Negative control samples consisted of diethyl pyrocarbonate-treated H_2O as no-template reaction. The relative intensity of internal

control and target products on gel was measured by luminescence with a DC-40 camera in combination with analysis software (Kodak, Rochester NY, USA). Subsequently, the relative concentration of MCP-1 or bFGF was divided by the relative concentration of GAPDH ([pg bFGF or MCP-1 / pg GAPDH] x 1000) to estimate the initial cytokine mRNA expression level in EMB.

Statistical analysis

We used the paired non-parametrical Wilcoxon signed rank test to define differences in MCP-1 and bFGF mRNA expression levels between EMB 0 and EMB 1. Correlations between mRNA expression levels of bFGF or MCP-1 and ischemic times or CsA trough levels were described using the non-parametrical Spearman correlation-coefficient (r). Differences in cytokine expression between patients with lower and higher acute rejection incidence were analysed with Fisher exact test in a cross-table. In all tests, p-values > 0.05 were considered statistically significant.

Results

MCP-1 and bFGF in association with ischemia

To study the influence of ischemia on the intragraft cytokine expression levels after clinical heart transplantation, we analysed the correlation between MCP-1 and bFGF mRNA expression levels and ischemic times (Table 1). Ischemic times ranged from 110 to 247 minutes (median 157.5 minutes) and did not correlate with intragraft MCP-1 mRNA expression in either EMB 0 (sampled during transplantation) ($r = -0.059$; $p = 0.80$) or EMB 1 (sampled after 1 week) ($r = 0.330$; $p = 0.15$). Similarly, ischemic times did not correlate with intragraft bFGF mRNA expression in either EMB 0 ($r = -0.174$; $p = 0.46$) or EMB 1 ($r = -0.419$; $p = 0.07$).

MCP-1 and bFGF in EMB 0 vs EMB 1

As a group ($n = 20$), significantly decreased mRNA expression levels of both MCP-1 and bFGF were measured in EMB 1 compared with EMB 0 from the same patients. Median relative MCP-1/GAPDH ratio decreased from 19.5 (range 2.2 to 705) to 3.9 (range 0.3 to 151) ($p = 0.006$, Figure 1A). Median relative bFGF/GAPDH ratio decreased from 21 (range 1.1 to 414) to 9 (range 0.1 to 381) ($p = 0.019$, Figure 1B).

Table 1: *Intragraft MCP-1 and bFGF mRNA expression levels in endomyocardial biopsies, as well as ischemic times, immunosuppressive therapy, and acute rejection episodes from 20 heart transplant patients*

Patient number	MCP/ GAPDH ratio in EMB 0	MCP/ GAPDH ratio in EMB 1	bFGF/ GAPDH ratio in EMB 0	bFGF/ GAPDH ratio in EMB 1	Ischemic time (min.)	Induction therapy (Yes/No)	Mean CsA t.l. until EMB 1 (ng/ml)	CsA t.l. at EMB 1 (ng/ml)	Number of AR during first year	GVD at one year (Yes/No)
1	22	1.7	43	7.0	145	N	357	230	1	N
2	7.1	2.0	5.2	11	165	N	237	230	3*	*
3	16	3.6	64	3.4	234	N	146	180	1	N
4	17	11	45	13	143	Y	227	290	2	N
5	5.0	0.8	121	30	134	Y	438	170	3	N
6	2.2	0.9	29	11	135	Y	240	340	3	Y
7	223	3.7	21	12	158	Y	260	370	2	N
8	705	0.7	243	18	128	Y	153	235	0	Y
9	56	39	414	28	228	Y	178	215	3	N
10	31	2.8	28	34	110	Y	290	280	0	N
11	22	25	6.8	21	121	Y	213	280	3	Y
12	14	151	42	381	162	Y	71	155	5	Y
13	86	17	18	0.1	218	Y	146	450	1	Y
14	178	71	7.6	0.5	134	Y	489	410	2	N
15	6.5	35	1.1	2.1	174	Y	268	300	2	nd
16	145	11	8.7	1.8	173	N	495	160	2	N
17	14	0.3	2.2	2.0	157	N	126	245	1	Y
18	77	16	21	6.0	200	N	171	165	1	Y
19	3.7	2.6	2.7	0.5	157	Y	108	195	0	Y
20	11	4.1	2.0	1.4	247	N	392	360	0	Y

MCP-1, monocyte chemoattractant protein-1; bFGF, basic fibroblast growth factor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; EMB 0, endomyocardial biopsy sampled during transplantation; EMB 1, endomyocardial biopsy samples during the first week after transplantation; CsA, cyclosporine; t.l., trough level; AR, acute rejection; GVD, graft vascular disease; nd: not determined; *patient died within the first year of acute rejection.

MCP-1 and bFGF in association with immunosuppression

To determine whether the down-regulation of both MCP-1 and bFGF in the first post-operative week was a direct effect of cyclosporine therapy, we calculated the correlation of intragraft cytokine mRNA expression with the CsA trough level at 1 week, as well as with the mean of all measured trough levels in the first week (on average 5 measurements per patient; range, 2 to 7) (Table 1 and Figure 2). Cyclosporine whole-blood trough levels in the simultaneously obtained peripheral blood samples with EMB 1 ranged from 155 to 450 ng/ml (median, 240 ng/ml) and did not correlate with intragraft mRNA expression of either MCP-1 ($r = 0.081$; $p = 0.74$, Figure 2A) or bFGF ($r = -0.332$; $p = 0.15$, Figure 2B). Mean CsA whole-blood trough levels in the period between EMB 0 and EMB 1 ranged from 71 to 495 ng/ml (median, 232 ng/ml) and did not correlate with either MCP-1 ($r = -0.005$; $p = 0.98$, Figure 2C) or bFGF mRNA expression ($r = -0.023$; $p = 0.92$, Figure 2D). Furthermore, we could find no difference in cytokine expression levels between patients who did or did not receive induction therapy with horse-ATG (Table 1).

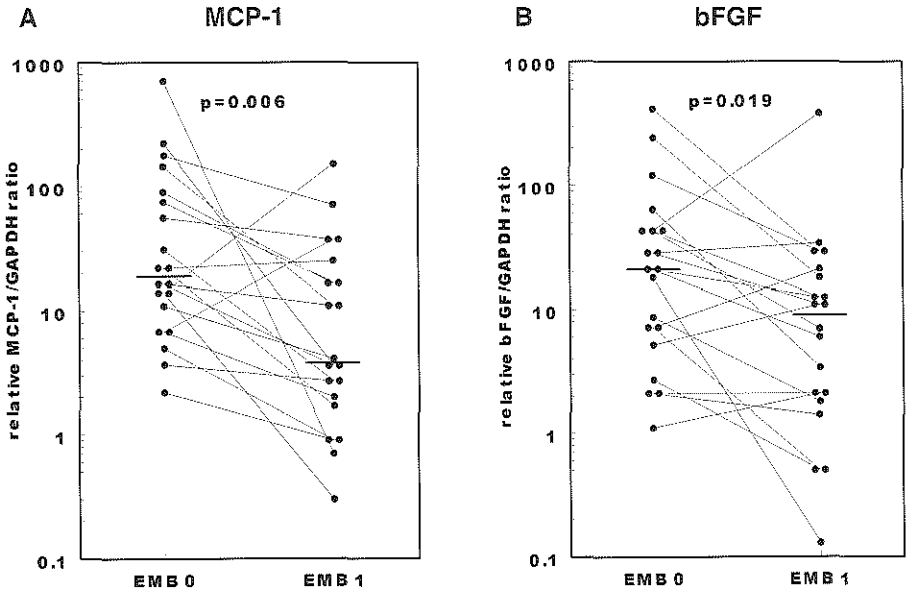

***Figure 1**: Monocyte chemoattractant protein (MCP-1) mRNA expression level (A) and basic fibroblast growth factor (bFGF) mRNA expression level (B) in endomyocardial biopsies obtained at 30 minutes (EMB 0) and in the first week post-transplant (EMB 1) from the same patients. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.*

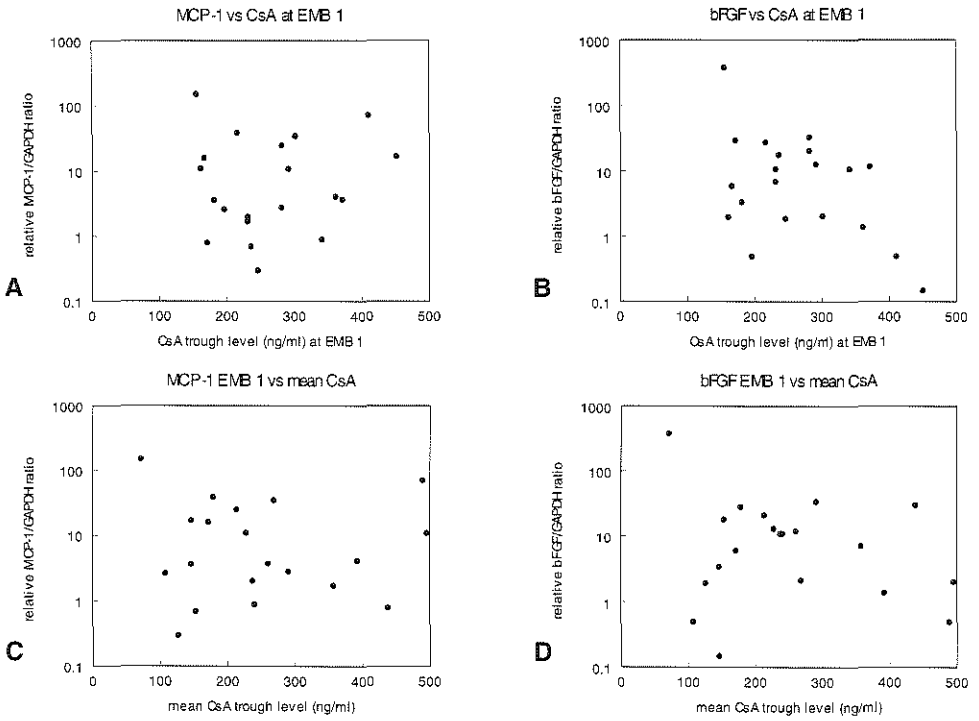

Figure 2: *Scatter plots of the monocyte chemoattractant protein (MCP-1) and basic fibroblast growth factor (bFGF) mRNA expression levels in the first post-operative week (EMB 1) vs cyclosporine (CsA) trough levels as measured at the day of biopsy sampling (A and B) and as mean of all measurements during the first week (C and D). GAPDH, glyceraldehyde 3-phosphate dehydrogenase.*

MCP-1 and bFGF in association with acute rejection and graft vascular disease

As shown in Figure 1, 5 of 20 patients had an increase in MCP-1 and/or bFGF mRNA expression during the first post-operative week: in 3 patients, both MCP-1 and bFGF mRNA expression levels rose, whereas in 2 patients, only bFGF mRNA expression levels were up-regulated. The increasing cytokine mRNA expression of MCP-1 and/or bFGF in the first week was associated with acute rejection later on. We found a high incidence of acute rejection episodes (>2 in the first year) in 3 of 5 (60%) patients with a rising MCP-1 and/or bFGF mRNA expression level in the first week post-transplant vs 3 of 15 (20%) patients with decreasing MCP-1 and/or bFGF mRNA expression levels ($p = 0.13$). Moreover, in patients with more than 2 acute rejection episodes in the first year, we measured significantly higher bFGF mRNA expression levels in EMB 1 compared with patients with a lower rejection incidence (median relative bFGF/GAPDH ratio, 24.5 (range 11 to 381) vs 2.8 (range, 0.1 to 34); $p = 0.009$, Figure 3B). We did not find this

difference for the MCP-1 mRNA expression levels (median relative MCP-1/GAPDH ratio, 13.5 (range 0.8 to 151) vs 3.9 (range, 0.3 to 71); p = 0.378, Figure 3A). Angiographic evidence of graft vascular disease at 1 year after transplantation was detected in 9 of 20 patients (Table 1). However, we found no association between MCP-1 or bFGF mRNA expression levels in EMB 0 or EMB 1 and the development of graft vascular disease in the first year.

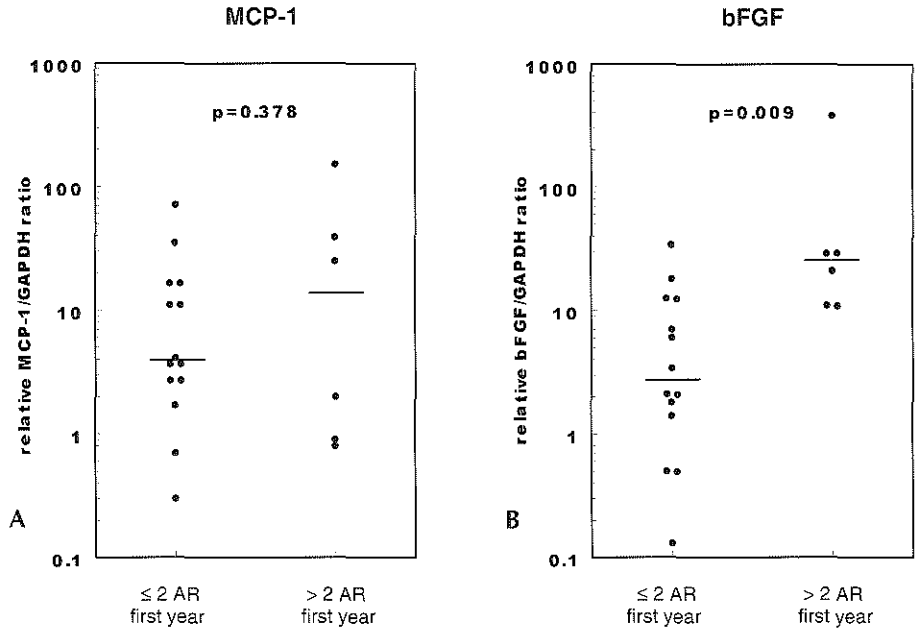

***Figure 3**: Monocyte chemoattractant protein (MCP-1) expression level (A) and basic fibroblast growth factor (bFGF) mRNA expression level (B) in patients with, respectively, ≤2 acute rejecions (AR) and >2 acute rejections in the first post-operative year. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.*

Discussion

In the present study, we monitored intragraft mRNA expression of chemokine MCP-1 and growth factor bFGF in the first week after clinical heart transplantation in relation to ischemia, immunosuppression, and rejection. Experimental studies, in the absence of immunosuppression, have shown that both allogeneic and non-allogeneic factors can trigger the release of cytokines, including MCP-1 and bFGF. Exposure to ischemia-reperfusion injury alone leads to up-regulation of both MCP-1 and bFGF protein expression in the myocardium of animals.[8,9,14] Several experimental studies have shown up-regulated MCP-1 and bFGF expression after transplantation accompanied by cellular influx and acute graft rejection.[5,10,16] Similarly, our clinical results demonstrate mRNA

expression of chemokine MCP-1 and growth factor bFGF in cardiac transplants early after transplantation. However, in contrast to the experimental studies, overall we showed a significant decrease of intragraft MCP-1 and bFGF mRNA expression levels in the first post-operative week. This pattern may also apply to other chemokines and growth factors, respectively, because their expression is triggered by an overlapping set of transcription factors. The combined influence of non-allogeneic events such as brain-death, ischemia, and (re)perfusion might be responsible for the initial high cytokine mRNA expression levels as measured during the transplantation procedure (EMB 0).[8,14,19,20]

Because down-regulation of both cytokines has occurred *in vitro* and *in vivo*, with immunosuppressive drugs, the decrease in the first week could, at least in part, be ascribed to immunosuppressive therapy. Cyclosporine and dexamethasone can down-regulate MCP-1 mRNA and protein expression in fibroblasts and endothelial cells.[21,22] In rats, peak bFGF mRNA expression at day 5 after limb transplantation could be inhibited by treatment with the immunophilin tacrolimus.[16] However, in the present study, we did not find a direct correlation between mRNA expression levels of MCP-1 or bFGF and (h-ATG) induction therapy or CsA trough levels. A number of factors could explain this discrepancy. First, recent reports indicate that monitoring CsA trough levels is not the most accurate indicator of total drug exposure.[23] Therefore, correlating the cytokine expression levels with the area under the curve or 2-hour peak levels might reveal another picture. Second, CsA trough levels were measured in peripheral blood, which does not parallel local concentration within the heart tissue (the site where cytokine mRNA expression levels were determined).[24] Third, in addition to CsA, patients received low-dose steroids, also shown to down-regulate cytokine mRNA expression levels.[25] And finally, individual differences may exist in the response to immunosuppression therapy as well as in cytokine expression levels (because of gene polymorphisms).[26]

Strikingly, most of the patients with increased MCP-1 and/or bFGF mRNA expression levels in the first week had a high incidence of acute rejection in the first year. Furthermore, in patients with a high incidence of acute rejection (>2 in the first year), significantly higher bFGF mRNA expression levels were measured in the first post-operative week. Recent studies have shown that a sub-set of human T cells can express FGF receptor and thereby can respond to FGF at inflammatory sites.[12,15] The authors hypothesize that when such T cells encounter their antigen at sites of increased FGF production, they receive sufficient costimulation for proliferation and expansion through stimulation of IL-2 production by activation of NF-kappaB. Furthermore, they show that the frequency of these FGF-responsive T cells increased in peripheral blood of cardiac allograft recipients compared with healthy controls. Together, these data suggest that

FGF is, in concordance with its function in tissue repair, involved in the early activation of the immune response after transplantation, which subsequently may lead to histologic signs of rejection in endomyocardial biopsies.

A recent study in human cardiac transplant recipients showed increased intragraft mRNA expression of FGF in the first year after transplantation in patients who developed graft vascular disease within 2 years post-transplant.[4] These authors speculate that the total quantity and cumulative exposure rather than the temporal pattern of FGF production contributes to the development of graft vascular disease. This might also explain our present finding that there was no association between MCP-1 or bFGF mRNA expression in the first week after transplantation and angiographic evidence of graft vascular disease at 1 year. However, one of the identified risk factors for development of graft vascular disease is (the incidence, severity and nature of) acute rejection.[1,2,18] Therefore, we speculate that patients with high bFGF levels in the first-week biopsy who experienced >2 acute rejection episodes in the first year might be predisposed for development of graft vascular disease at a later stage.

In summary, our clinical data show, in contrast to non-immunosuppressed experimental studies, rapid down-regulation of intragraft mRNA expression of both MCP-1 and bFGF in the first post-operative week after clinical heart transplantation. For patients in whom the initial intragraft MCP-1 and/or bFGF mRNA expression level increased, we found a high incidence of acute rejection. Therefore, we speculate that patients who fail to down-regulate the intragraft cytokine mRNA expression shortly after transplantation are at higher risk for acute rejection episodes.

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CHAPTER 3A

INTRAGRAFT PDGF-A AND TGF-β1 DURING THE DEVELOPMENT OF ACCELERATED GRAFT VASCULAR DISEASE AFTER CLINICAL HEART TRANSPLANTATION

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Transplant Immunology 1999; 7 (4): 201 - 205.

Abstract

This study was to determine whether the growth factors platelet-derived growth factor-A (PDGF-A) and transforming growth factor-beta1 (TGF-β1) contribute to the development of graft vascular disease (GVD) after clinical heart transplantation. We analysed intragraft PDGF-A and TGF-β1 messenger RNA (mRNA) expression levels by competitive template reverse transcriptase polymerase chain reaction (RT-PCR). Endomyocardial biopsies (EMB) were obtained at 1 and 9 months post-transplant from cardiac allograft recipients with (n = 11) and without (n = 11) angiographic evidence of GVD at 1 year. In 1-month EMB, comparable TGF-β1 mRNA levels were found in patients with and without GVD at 1 year (p = 0.84, Mann-Whitney *U* test). In contrast, in 9-months EMB during the development of GVD, intragraft mRNA levels of both PDGF-A (p = 0.08) and TGF-β1 (p = 0.03) were higher in patients with GVD after the first year compared to patients without GVD. These results suggest that intragraft PDGF-A and TGF-β1 play a role in the pathogenesis of accelerated GVD after clinical heart transplantation.

Introduction

Long term survival after clinical heart transplantation is influenced by graft failure due to graft vascular disease (GVD).[1,2] GVD is characterized by progressive intimal thickening as a result of smooth muscle cell (SMC) proliferation along the entire length of the epicardial as well as the intramyocardial coronary arteries.[3] A number of allogen-dependent and allogen-independent factors has been implicated in the pathogenesis of GVD.[4] In the response-to-injury model a common pathway has been proposed to explain the contribution of various identified risk factors. Brain death, ischemia, reperfusion, donor specific human leucocyte antigen (HLA) antibodies, number and nature of acute rejection episodes, and cytomegalovirus (CMV) infection may all damage the endothelium of the graft leading to early inflammatory events including the secretion of growth factors.[5,6] Recently, we found an association between donor age, ischemic time, intragraft IL-2 mRNA expression during the first acute rejections, as well as IFN-γ production by graft infiltrating lymphocytes, and the development of GVD at one year after clinical heart transplantation.[7,8] Because it has been reported that activated vascular cells can secrete platelet-derived growth factor-A (PDGF-A) and transforming growth factor-beta (TGF-β),[9,10] we now speculate that PDGF-A and TGF-β1 production may function as a linking factor between these early antigen-dependent and -independent factors and the development of GVD. PDGF-A and TGF-β1 are produced by many different cell types including platelets, macrophages, lymphocytes, activated endothelial cells, vascular SMC and myocytes.[11,12] The effect of TGF-β1 on cell proliferation is either inhibiting or stimulating depending on its concentration and the

local presence of other growth factors. In addition, TGF-β1 is able to induce the production of PDGF-A and the expression of its receptor.[13] Both growth factors are often associated with the development of GVD because of their presence in atherosclerotic lesions and their attractive and mitogeneic influence on SMC in vitro. Indeed, several experimental and clinical studies have shown that PDGF-A and TGF-β1 are present in cardiac allografts with evidence of end-stage GVD.[14,15,16,17,18] Recently, an association between GVD and TGF-β1 gene-polymorphism in heart transplant patients has been reported.[19,20] Homozygous TGF-β1 genotype in recipients and persistent expression of TGF-β1 staining in EMB was correlated with increased risk of GVD.

Objective

In the present study, we determined intragraft mRNA expression levels of PDGF-A and TGF-β1 by reverse transcriptase polymerase chain reaction (RT-PCR) in a clinical situation before the diagnosis of GVD was assessed.

Material and methods

Patients

We studied intragraft mRNA expression of PDGF-A and TGF-β1 in EMB from heart transplant patients with GVD at 1 year (n = 11). Patients who remained free from GVD in the first year served as controls (n = 11). Patients underwent transplantation between April 1992 and August 1994. GVD was defined as all vascular wall changes, including minor irregularities, as assessed by visual analysis of coronary angiograms.[21] Control patients were matched for age, gender, original heart disease, date of transplantation and immunosuppressive induction therapy. Maintenance immunosuppressive therapy consisted of CsA and low-dose steroids. Monitoring of rejection was performed by EMB and graded according to the guidelines of the International Society for Heart and Lung Transplantation.[22] For the purpose of this study rejection was defined as rejection requiring therapy (grade ≥ 3A). Baseline characteristics of the patients at the time of transplantation are summarized Table 1. From each patient, we studied mRNA expression of PDGF-A and TGF-β1 in EMB sampled at approximately 1 and 9 months after heart transplantation, thus before the diagnosis of GVD was assessed during the development of GVD.

Messenger RNA isolation and cDNA synthesis

After sampling, the EMB specimens were snapfrozen in liquid nitrogen and stored at -80°C until mRNA isolation. Messenger RNA extraction and transcription was performed

as described previously.[7] Briefly, snap-frozen EMB specimens were homogenized in 500 μl 4 M guanidinium-isothiocyanate in the presence of 20 μg poly A (Boehringer, Mannheim, Germany). The solution was extracted once with phenol, phenol-chloroform-isoamyl alcohol (25:24:1) and chloroform-isoamyl alcohol (49:1), respectively. Total RNA was precipitated with 600 μl 2-propanol and 35 μl 3 M sodium acetate (pH 5.2) at -20°C for 18 hours. Precipitates were centrifuged at 10,000 *g* at 4°C and washed once with 500 μl icecold 80% ethanol. Air-dried pellets were resuspended in 50 μl diethyl pyrocarbonate-treated H_2O. Total RNA was denatured for 5 minutes at 80°C and then chilled on ice. First-strand complementary DNA (cDNA) synthesis was performed in 2 x 25 μl of the isolated RNA with 1.25 μg hexanucleotides (Promega Corporation, Madison WI, USA) and transcribed with 500 U Moloney murine leukemia virus reverse transcriptase (MMLV-RT; Gibco-BRL, Gaithersburg MD, USA) at 42°C for 90 minutes in a total volume of 50 μl. The reaction mixture consisted of 10 μl of 5x MMLV-RT buffer (250 mM Tris-HCl pH 8.3, 15 mM $MgCl_2$, 375 mM KCl), 2.5 μl dNTP (10 mM), 200 U of RNAsin (Promega) and 5 μl (0.1 M) dithiothreitol.

Table 1: *Baseline characteristics of the study group of 22 heart transplant recipients.*

Characteristic:	GVD -	GVD+	p-value
number of patients (n)	11	11	
mean age recipient (years ± SD)	46.2 ± 17	55.7 ± 6.6	p = 0.11[a)]
mean age donor (years ± SD)	25.9 ± 8	34.2 ± 7.5	p = 0.02[a)]
gender recipient (male/female)	10/1	9/2	
ischemic time (minutes ± SD)	140.6 ± 24.2	189.2 ± 36.7	p = 0.002[a)]
primary disease:			
ischemic heart disease (n)	6	8	
cardiomyopathy (n)	5	3	
induction therapy:			
OKT3 (n)	8	7	
BT563 (n)	3	3	
ATG (n)	0	1	
mean number of AR in the first year (n ± SD)	2.9 ± 1.6	3.3 ± 1.6	p = 0.59[a)]
IL-2 mRNA present during first AR (n)	3	8	p = 0.03[b)]

GVD, graft vascular disease; n, number; SD, standard deviation; AR, acute rejection; [a)]Student's *t* test; [b)]Fischer's exact test.

Competitive template RT-PCR for PDGF-A, TGF-β1, and GAPDH

To estimate the relative initial amount of PDGF-A and TGF-β1 in EMB, a competitive template RT-PCR assay was used and a comparison was made against the house-keeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The latter gene is assumed to be expressed at a constant level in EMB. Sequence-specific primers were used for quantitative amplification of PDGF-A (sense: 5'-aga.agt.cca.ggt.gag. gtt.aga.gga.gca.t-3'; anti-sense: 5'-ctg.ctt.cac.cga.gtg.cta.caa.tac.ttg.ct-3'), TGF-β1 (sense: 5'-gcc.ctg.gac. acc.aac.tat.tgc-3'; anti-sense: 5'-gct.gca.ctt.gca.gga.gcg.cac-3'), and GAPDH (sense: 5'-ggt.gaa.ggt.cgg.agt.caa.cg-3'; anti-sense: 5'-caa.agt.tgt.cat.gga.tga.cc-3'). To obtain a standard curve for PDGF-A, TGF-β1, and GAPDH, known amounts of internal control fragment were added in different dilutions to constant amounts of sample cDNA for competitive co-amplification. The internal control was designed to generate a PCR product of a smaller size to allow differentiation between the amplified target and the internal control. Sample cDNA and internal control, 5 μl each, were added to a 90 μl PCR mixture containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dATP, dCTP, dTTP, dGTP, 2 U Ampli Taq Gold (Perkin-Elmer, Norwalk CT, USA) and 50 pmol of sense and anti-sense sequence-specific primers. Each reaction mixture was overlaid with 100 μl mineral oil (Sigma, St. Louis MO, USA) before PCR reaction in a DNA thermal cycler model 480 (Perkin-Elmer) under the following conditions. After a 10 minute 94°C denaturation step, samples underwent 40 cycles of 1 minute denaturation at 94°C, 2 minutes annealing at 60°C, and 3 minutes extension at 72°C for 3 minutes. The last cycle was extended with 7 minutes at 72°C. Positive control samples were produced by stimulating 10^6 human spleen cells with 1% phytohemagglutinin-M (Difco, Detroit MI, USA) for 24 hours at 37°C. Messenger RNA from this positive control was extracted as described above. Negative control samples consisted of diethyl pyrocarbonate-treated H_2O as no-template reaction.

After amplification, 16 μl PCR product was electrophoresed through 2% agarose gel, and the amount of products by the internal control and targets were determined for each individual reaction. The relative ethidium bromide intensity on gel was measured by luminescence with a DC-40 camera in combination with analysis software (Kodak, Rochester NY, USA). The logarithm of the ratio target/internal control was graphed as a function of the logarithm of the internal molar amount of the standard. At ratio 1 (equilibration point) the starting concentration of target mRNA before PCR is assumed to be equal to the known starting concentration of the competing internal control. Subsequently, the relative concentrations of PDGF-A and TGF-β1 were divided by the relative concentration of GAPDH to obtain a ratio for the initial PDGF-A and TGF-β1 mRNA expression level in EMB.

Statistical analysis

The non-parametric Mann-Whitney *U* test was used to define differences in PDGF-A and TGF-β1 mRNA expression levels in EMB between patients with and without GVD at one year. P-values ≤ 0.05 were considered to be statistically significant.

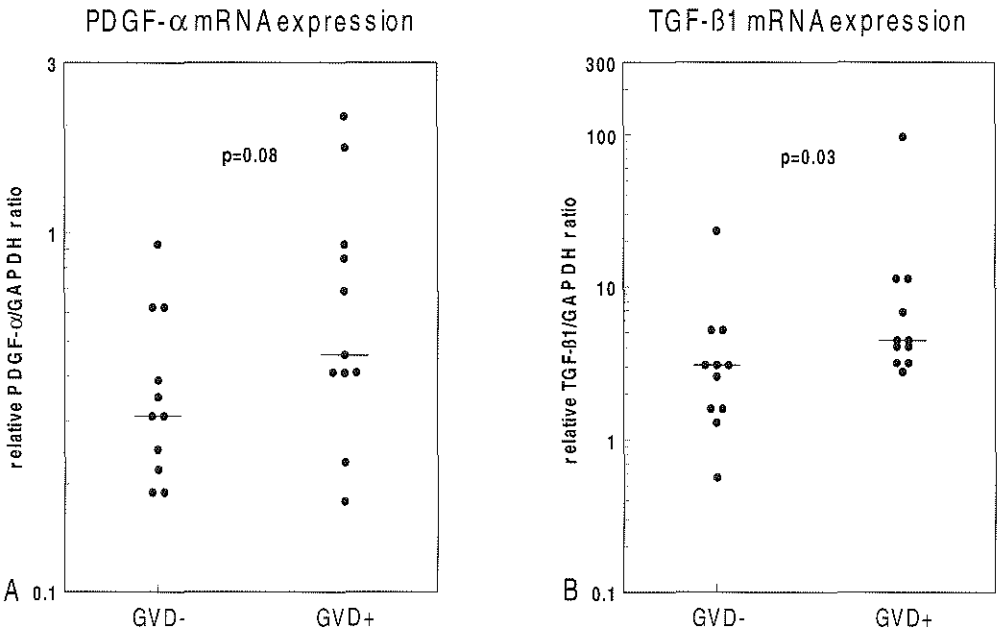

Figure 1: *PDGF-A mRNA expression (A) and TGF-β1 mRNA expression (B) in 9-months EMB obtained from patients without GVD (-) and with GVD (+) one year after transplantation. Median PDGF-A/GAPDH mRNA expression in GVD- and GVD+ was 0.31 vs 0.46 (p = 0.08; Mann-Whitney U test) and median TGF-β1/GAPDH mRNA expression in GVD- and GVD+ was 3.1 vs 4.4 (p = 0.03).*

Results

Patients with GVD at 1 year after transplantation received cardiac allografts from older donors (p = 0.02, Table 1), and exposed to longer ischemic times (p = 0.002, Table 1) compared to control patients without GVD at 1 year. The mean number of acute rejections in the first year was not different between patients with or without GVD at 1 year; however, patients with GVD had more first rejection biopsies which were positive for IL-2 mRNA (p = 0.03, Table 1).[7] At 1 month after transplantation we did not find a difference in TGF-β1 mRNA expression levels between patients with and without GVD at 1 year. The median relative TGF-β1/GAPDH ratio for patients with GVD was 5.6

(range 1.0 to 26.9) vs 4.0 (range 2.2 to 9.4) for patients without GVD ($p = 0.84$). In contrast, at 9 months after transplantation the intragraft PDGF-A and TGF-β1 mRNA expression levels were higher in patients who developed GVD in the first year. The median relative PDGF-α/GAPDH ratio in patients with GVD was 0.46 (range 0.18 to 2.13) vs 0.31 (range 0.18 to 0.93) for patients without GVD ($p = 0.08$, Figure 1a) and the relative TGF-β1/GAPDH median ratio was 4.4 (range 2.8 to 96.7) vs 3.1 (range 0.57 to 23.3) respectively ($p = 0.03$, Figure 1b). In general, TGF-β1 mRNA expression levels were 10 times higher compared with the expression levels of PDGF-A mRNA.

Discussion

We investigated whether the intragraft expression of PDGF-A and TGF-β1 is associated with accelerated development of GVD after clinical heart transplantation. These two growth factors could be involved in the cascade of early inflammatory events induced by various risk factors (donor age, ischemic time, rejection) leading to GVD. We found higher PDGF-A and TGF-β1 mRNA expression levels in EMB obtained at 9 months posttransplant from patients developing accelerated GVD. As reported earlier, this patient group received older donor hearts exposed to longer ischemic times. The number of acute rejection episodes during the fist year was comparable, but EMB sampled during the first acute rejection were more often positive for IL-2 mRNA in patients with accelerated GVD.[7]

Production of both PDGF-A and TGF-β1 by different cell types has been demonstrated in vitro as well as in vivo under experimental conditions. For example, pooled human arterial or aortic endothelial cells in culture secrete PDGF protein and show increased PDGF-A and TGF-β1 mRNA expression upon stimulation with allogeneic lymphocytes.[23,24] In a rat aorta transplantation model, the number of PDGF-A and TGF-β1 staining cells was increased by allogen-independent and -dependent stimuli.[25]

The presence of PDGF and TGF-β protein has often been associated with end stage GVD in rat models.[16,17,18] In humans, an immunohistochemical study described more prevalent PDGF-A staining in EMB from allograft recipients with end stage GVD compared to recipients without GVD.[15] It has been reported recently that heart transplant patients with a homozygous TGF-β1 genotype show excessive TGF-β1 immunostaining in their EMB and are at higher risk for the development of GVD.[19,20] In the present study, higher intragraft PDGF-A and TGF-β1 mRNA expression levels were found in patients developing GVD who received older donor hearts with longer ischemic times and experienced a higher number of IL-2 positive first acute rejections. This finding suggests that intragraft PDGF-A and TGF-β1 production, which might

indirectly be stimulated as a result of exposure to early risk factors, contributes to the accelerated development of GVD after clinical heart transplantation.

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CHAPTER 3B

CYCLOSPORINE A DOWNREGULATES INTRAGRAFT PLATELET DERIVED GROWTH FACTOR-A MRNA EXPRESSION AFTER CLINICAL HEART TRANSPLANTATION

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Transplantation Proceedings 2001; 33: 2241 - 2243.

Introduction

Transforming growth factor-β1 (TGF-β1) and platelet derived growth factor-A (PDGF-A) are multifunctional cytokines. TGF-β1 stimulates cell proliferation, regulates tissue repair and has immune-modulating properties. It is secreted by various celltypes including endothelial cells, smooth muscle cells and immune competent cells such as activated T-cells and monocytes/macrophages.[1] PDGF-A is a peptide mitogen that can be produced by platelets, macrophages, endothelial cells, and vascular smooth muscle cells and plays an important role in wound healing by stimulating cell proliferation and by inducing migration of leukocytes to injured tissue.[2] TGF-β1 and PDGF-A are thought to be involved in the pathogenesis of chronic rejection after organ transplantation by triggering smooth muscle cell migration and proliferation, inhibition of epithelial regeneration, and induction of fibroblast proliferation, ending in fibrosis.[3,4] Recently, we reported higher mRNA expression levels of both TGF-β1 and PDGF-α in cardiac transplant patients during the development of accelerated graft vascular disease (GVD),[5] while others described the presence of both growth factors in end-stage GVD lesions.[6,7] The immunosuppressive agent cyclosporine A (CsA) has been reported to stimulate TGF-β1 production. In vitro studies have shown that CsA up-regulates TGF-β1 messenger RNA (mRNA) expression as well as TGF-β1 protein production by cultured human T cells and human mesangial cells.[8,9] In addition, CsA has been demonstrated to increase serum TGF-β1 concentrations both in animal models and renal transplant recipients.[10,11] However, the reliability of TGF-β measurements in serum has been questioned due to limitations of the analytical technique.[12] Studies on the influence of CsA on PDGF-A are few, but in an experimental rat model for chronic rejection an inhibitory activity of CsA on the production of PDGF-A protein and PDGF-A receptor has been demonstrated.[13] In the present study, we analysed whether CsA blood levels correlate with the development of GVD and with intragraft TGF-β1 or PDGF-A mRNA expression levels after clinical heart transplantation.

Patients and methods

Patients

We studied endomyocardial biopsies (EMB) and whole blood samples collected at 9 months after transplantation from 10 patients with GVD at 1 year and 10 control patients who remained free from GVD in the first year. Patients underwent transplantation between August 1992 and August 1994. GVD was defined as all vascular wall changes, including minor irregularities, as assessed by visual analysis of coronary angiogram.[14] Maintenance immunosuppressive therapy consisted of CsA and low-dose steroids. CsA trough levels were routinely monitored in whole blood (EDTA)

by Cyclo-Trac-SP radioimmunoassay (Incstar, Stillwater, Minn, USA). Monitoring of rejection was performed by EMB and graded according to the guidelines of the International Society for Heart and Lung Transplantation. [15] In EMB, without signs of acute rejection episodes requiring additional anti-rejection therapy (ISHLT rejection grade 0, 1 or 2), we measured mRNA expression levels of PDGF-A and TGF-β1.

RT-PCR

Total RNA was extracted from snapfrozen EMB, and subsequently cDNA was synthesized with random primers using techniques previously reported in detail. [16] Aliquots cDNA (representing 1/20 EMB) were directly used for PCR amplification using sequence-specific primers.[5] To estimate the relative initial amount of functional TGF-β1 and PDGF-A mRNA in EMB, a competitive template RT-PCR assay was used and a comparison was made against the housekeeping gene GAPDH. To obtain a standard curve for PDGF-A, TGF-β1, and GAPDH, known amounts of internal control fragment were added in different dilutions to constant amounts of sample cDNA for competitive coamplification. The internal control was designed to generate a PCR product of a smaller size to allow differentiation between the amplified target and the internal control. The relative intensity of internal control and target products on gel was measured by luminescence with a DC-40 camera in combination with analysis software (Kodak, Rochester NY, USA). Subsequently, the relative concentrations of PDGF-A and TGF-β1 were divided by the relative concentration of GAPDH to estimate the initial cytokine mRNA expression level in EMB.

Statistics

The non-parametric Mann-Whitney *U* test was used to define differences in CsA trough levels between patients with and without GVD at 1 year. The correlation between CsA trough levels and TGF-β1 or PDGF-A mRNA expression levels was described by the nonparametric Spearman's correlation-coefficient (r). p values ≤ 0.05 were considered to be statistically significant.

Results

In general, intragraft TGF-β1 RNA expression levels were 10 times higher than intragraft expression levels of PDGF-A (median TGF-β1/GAPDH ratio 4.1, range 0.6 to 96.7 and median PDGF-A/GAPDH ratio 0.4, range 0.18 to 2.13). CsA whole blood trough levels at 9 months posttransplant ranged from 140 to 540 ng/ml. We observed that these CsA trough levels were not different between patients who did or did not develop GVD in

the first year (median 250 ng/ml, range 140 to 410 ng/ml vs. 275 ng/ml, range 190 to 540 ng/ml; p = 0.47).

To determine the effect of CsA on intragraft PDGF-A and TGF-β1 levels, we calculated the Spearman's correlation coefficient between CsA trough levels and the expression levels of PDGF-A and TGF-β1 in EMB at 9 months posttransplant. No correlation could be found between CsA whole blood trough levels and intragraft TGF-β1 mRNA levels (r = -0.07, p = 0.76, figure 1A). However, there was a significant inverse correlation between CsA whole blood trough levels and intragraft PDGF-A mRNA levels (r = -0.56, p = 0.01, figure 1B).

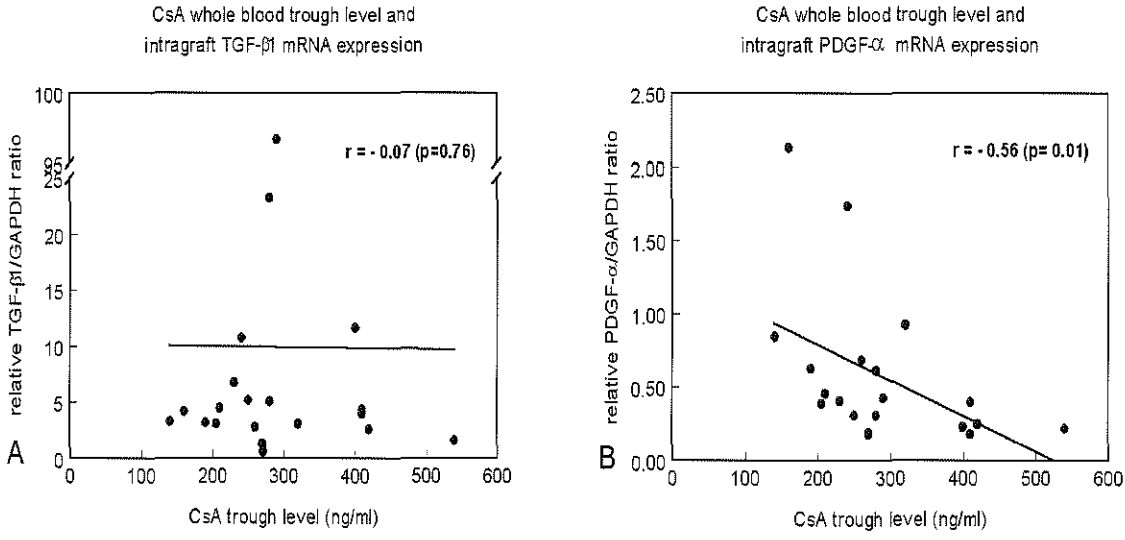

Figure 1: *Correlation between Cyclosporine A whole blood trough levels and intragraft mRNA expression levels of TGF-β1 (A) and PDGF-A (B) at 9 months posttransplant.*

Discussion

The expression of TGF-β1 and PDGF-A have often been associated with the development of chronic rejection after organ transplantation. We recently have shown higher mRNA expression levels of both TGF-β1 and PDGF-A in patients who develop accelerated GVD.[5] Despite the useful immunosuppressive properties of CsA in the treatment of transplant recipients, a number of investigators have suggested that CsA increases TGF-β1 expression and therefore may be a risk factor for the development of chronic rejection in renal allografts.[8,17] Conflicting results have been reported concerning the relationship between CsA and GVD.[18] Our results in cardiac allograft

recipients did not show differences in 9-months CsA trough levels between patients with and without development of GVD in the first year. However, we diagnosed GVD by visually assessment of coronary angiogram. Quantitative measurement by intravascular ultrasound (IVUS) is a more sensitive diagnostic tool and might reveal another picture of the association between CsA trough levels and GVD.

A clinical study in pre-transplant patients with end stage renal failure showed a significant increase of TGF-β1 mRNA and TGF-β1 protein in peripheral blood after CsA therapy.[19] However, these patients were tested before transplantation, thereby lacking the influence of allogeneic factors on TGF-β1 production. In the present study, we analysed CsA trough levels and intragraft TGF-β1 and PDGF-A mRNA expression after clinical heart transplantation. In our patients, no correlation was observed between CsA whole blood trough levels and intragraft TGF-β1 mRNA levels. These results are in line with findings in renal allograft recipients where CsA blood levels failed to correlate with TGF-β1 staining in biopsies, TGF-β1 plasma levels, or kidney function.[11,20,21] In contrast, we demonstrated a significant inverse correlation between CsA trough levels and intragraft mRNA expression of PDGF-A. The same effect of CsA on PDGF-A has also been observed in an experimental transplantation model in rats, where protein expression of PDGF-α was reduced in the media and intima of coronary arteries/arterioles in the high dose CsA treatment group.[13] This suggests that CsA might have an effect on the development and/or progression of GVD after heart transplantation.

In summary, our results show that CsA blood levels at 9 months are neither directly associated with development of accelerated GVD nor correlated with intragraft mRNA levels of TGF-β1, whereas there is a significant inverse relation with PDGF-A mRNA levels in the graft. This suggests, both in patients with and without development of GVD, a trough-level dependent downregulating effect of CsA on intragraft PDGF-A mRNA expression after clinical heart transplantation.

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CHAPTER 4

DIFFERENTIAL INTRAGRAFT CYTOKINE mRNA PROFILES DURING REJECTION AND REPAIR OF CLINICAL HEART TRANSPLANTS

--- A LONGITUDINAL STUDY ---

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Transplant International; in press.

Abstract

After clinical heart transplantation, ischemia, acute rejection, and repair mechanisms can trigger the upregulation of cytokines. To investigate the cytokine profile early after transplantation, we monitored mRNA expression levels of TNF-α, MCP-1, TGF-β, PDGF-A, and bFGF by RT-PCR in serial endomyocardial biopsies (n = 123) from sixteen cardiac allograft recipients during the first three postoperative months. In the first month, mRNA expression levels of MCP-1, TNF-α, TGF-β, and bFGF were significantly higher compared to the period thereafter (acute rejection episodes excluded). Acute rejection (ISHLT rejection grade > 2) was strongly associated with the level of TNF-α mRNA. After acute rejection episodes, rising mRNA expression levels of PDGF-A and bFGF were found. The association between TNF-α mRNA and acute rejection reflects the importance of this cytokine in allogeneic responses. Elevated growth factor expression levels indicate repair responses after tissue damage due to either the transplantation procedure (surgery, ischemia, reperfusion) or acute allograft rejection.

Introduction

Cytokines are regulatory proteins that play a central role in the anti-donor immune response.[1,2] Their release by vascular endothelial cells, cardiac myocytes and/or inflammatory cells can be triggered by various allo-antigen (in)dependent factors, such as brain death, ischemia, reperfusion and acute rejection episodes.[3,4] It has been suggested that early cytokine responses after transplantation can influence later graft outcome. For example, elevated levels of IL-6 and TNF-α early after clinical heart transplantation corresponded to reduced survival,[5] and elevated growth factor expression (eg TGF-β, PDGF-A, bFGF) has been linked to unrestricted repair responses and development of chronic allograft rejection both in experimental and in clinical studies.[2,6] Furthermore, increased pro-inflammatory cytokine and chemokine mRNA and protein levels have been found in association with acute allograft rejection.[2,7] Thus, information regarding the kinetics of cytokine expression after clinical transplantation can lead to a better understanding in events of graft adaptation and rejection. However, serial intragraft cytokine measurements in the recent clinical heart transplant setting are few.[8,9]

The aim of the present study was to identify changes in cytokine profiles in time and during acute allograft rejection early after clinical heart transplantation. Therefore, we analysed serial patterns of intragraft cytokine mRNA expression during the first three postoperative months in cardiac allograft recipients. We selected a broad range of cytokines, which can be released by activated endothelial cells, cardiac myocytes, and infiltrated mononuclear cells: tumor necrosis factor-α (TNF-α), monocyte chemoattractant protein-1 (MCP-1), transforming growth factor-β (TGF-β), platelet derived growth factor (PDGF-A), and basic fibroblast growth factor (bFGF). The pro-inflammatory cytokines TNF-α and MCP-1 play a significant role in the regulation and recruitment of cells that participate in inflammatory responses.[10,11] The growth factors TGF-β, PDGF-A and bFGF are polypeptides with potent mitogenic activity for fibroblasts, smooth muscle cells and endothelial cells and are important in tissue repair processes.[3,12]

Patients and methods

Patients

We studied 16 consecutive cardiac allograft recipients who were transplanted between November 1997 and October 1998. Maintenance immunosuppressive therapy consisted of cyclosporin A and low dose steroids. Serial endomyocardial biopsies (EMB) obtained from the right ventricle were studied during the first three months post-transplant. Timing of surveillance biopsies in this period was weekly during the first 6 weeks and biweekly during the following 8 weeks. During routine biopsy sampling, an additional biopsy was harvested for cytokine studies after informed consent of the patients. In total, 123 biopsies (on average 8 time points per patient) were available for cytokine analysis. In addition, "time-zero" biopsies were sampled from 10 patients during the transplantation procedure. Acute rejection was diagnosed by histological assessment of myocardial biopsies and graded according to the guidelines of the International Society for Heart and Lung Transplantation (ISHLT).[13] Patients with ISHLT rejection grade >2 were considered to have an acute rejection and received additional immunosuppressive treatment.

Cytokine mRNA detection by quantitative RT-PCR

Competitive reverse transcriptase polymerase chain reaction (RT-PCR) was used for quantitative measurement of TNF-α, MCP-1, TGF-β, PDGF-A, bFGF, and the constitutively expressed housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Total RNA was extracted from snapfrozen EMB, and complementary DNA (cDNA) was synthesized with random primers as described previously in detail.[14] Aliquots of cDNA were directly used for PCR amplification, using sequence-specific primers for TNF-α (sense: 5'-GAG-TGA-CAA-GCC-TGT-AGC-CCA-TGT-TGT-AGC-A-3', antisense: 5'-GCA-ATG-ATC-CCA-AAG-TAG-ACC-TGC-CCA-GAC-T-3'), MCP-1 (sense: 5'-TAG-CAG-CCA-CCT-TCA-TTC-C-3', anti-sense: 5'-TTC-CCC-AAG-TCT-CTG-TAT-CT-3'), TGF-β (sense: 5'-GCC-CTG-GAC-ACC-AAC-TAT-TGC-3'; anti-sense: 5'-GCT-GCA-CTT-GCA-GGA-GCG-CAC-3'), PDGF-A (sense: 5'-AGA-AGT-CCA-GGT-GAG-GTT-AGA-GGA-GCA-T-3'; anti-sense: 5'-CTG-CTT-CAC-CGA-GTG-CTA-CAA-TAC-TTG-CT-3'), bFGF (sense: 5'-GGC-TTC-TTC-CTG-CGC-ATC-CA-3', anti-sense: 5'-GCT-CTT-AGC-AGA-CAT-TGG-AAG-A-3'), and GAPDH (sense: 5'-GGT-GAA-GGT-CGG-AGT-CAA-CG-3'; anti-sense: 5'-CAA-AGT-TGT-CAT-GGA-TGA-CC-3'). PCR conditions were 10-minute 94°C denaturation, followed by 40 cycles of 1-minute denaturation at 94°C, 2-minute annealing at optimal temperatures for TNF-α (60°C), MCP-1 (56°C), TGF-β (60°C), PDGF-A (60°C), bFGF (58°C), or GAPDH (60°C), and 3-minute extension at 72°C, prolonged for 7 minutes during the last cycle. Positive control samples were produced by messenger RNA extraction and cDNA synthesis from 10^6 human spleen cells stimulated with 1% phytohemagglutin-M (PHA; Difco, Detroit MI, USA) for 24 hours at 37°C. Negative control samples consisted of diethyl pyrocarbonate-treated H_2O as no-template reaction.

To quantify the initial amount of functional cytokine mRNA in EMB, a competitive template RT-PCR assay was used in which known amounts of specific internal control fragment in different dilutions were added to constant amounts of sample cDNA for competitive co-amplification. We designed the internal controls for each cytokine to generate a smaller PCR product to allow differentiation between the amplified target and the internal control. The intensity of internal control and target products on gel was measured by luminescence with a DC-40 camera in combination with analysis software (Kodak, Rochester NY, USA). The relative concentration of cytokine mRNA was divided

by the relative concentration of GAPDH to indicate the initial cytokine mRNA expression level in EMB. EMB negative for GAPDH mRNA expression were excluded from further analysis.

Statistics

Differences in median cytokine mRNA expression levels between EMB sampled during the first month and during the second/third month post-transplant, or EMB with and without histologically proven acute rejection, were analysed by non-parametric Mann-Whitney U test. Correlations of ischemic time with cytokine mRNA levels were calculated by Pearson's correlation coefficient (r) after log-transformation of the cytokine mRNA ratios. Associations with p-values < 0.05 were considered statistically significant.

Results

To determine the relationship between cytokine mRNA expression levels and time, without the influence of acute rejection on cytokine mRNA expression, we excluded the biopsies sampled during and three weeks following an acute rejection episode ($n = 30$). Figure 1 shows the median (and 25^{th} and 75^{th} percentile) of the cytokine mRNA expression levels in the remaining serial biopsies. Peak cytokine mRNA expression was measured in "time-zero" biopsies for MCP-1, bFGF and PDGF; in the first week for TNF-α, and bFGF; and in the third week for TNF-α, MCP-1, TGF-β, and bFGF (Figure 1). Grouping of the time-points revealed that the expression levels of TNF-α, MCP-1, TGF-β, and bFGF mRNA were significantly higher in the first post-operative month (including "time-zero" biopsies) compared to the period thereafter (Figure 2). The median cytokine/GAPDH mRNA ratio in the first month vs 2-3 months posttransplant were 13.8 vs 3.4 for MCP-1 ($p < 0.0001$), 23.0 vs 8.6 for TNF-α ($p = 0.007$), 1.6 vs 0.8 for TGF-β ($p = 0.002$), and 2.5 vs 1.5 for bFGF ($p = 0.006$). The expression level of PDGF-A was higher in "time-zero" biopsies, but remained at a constant expression level during follow up.

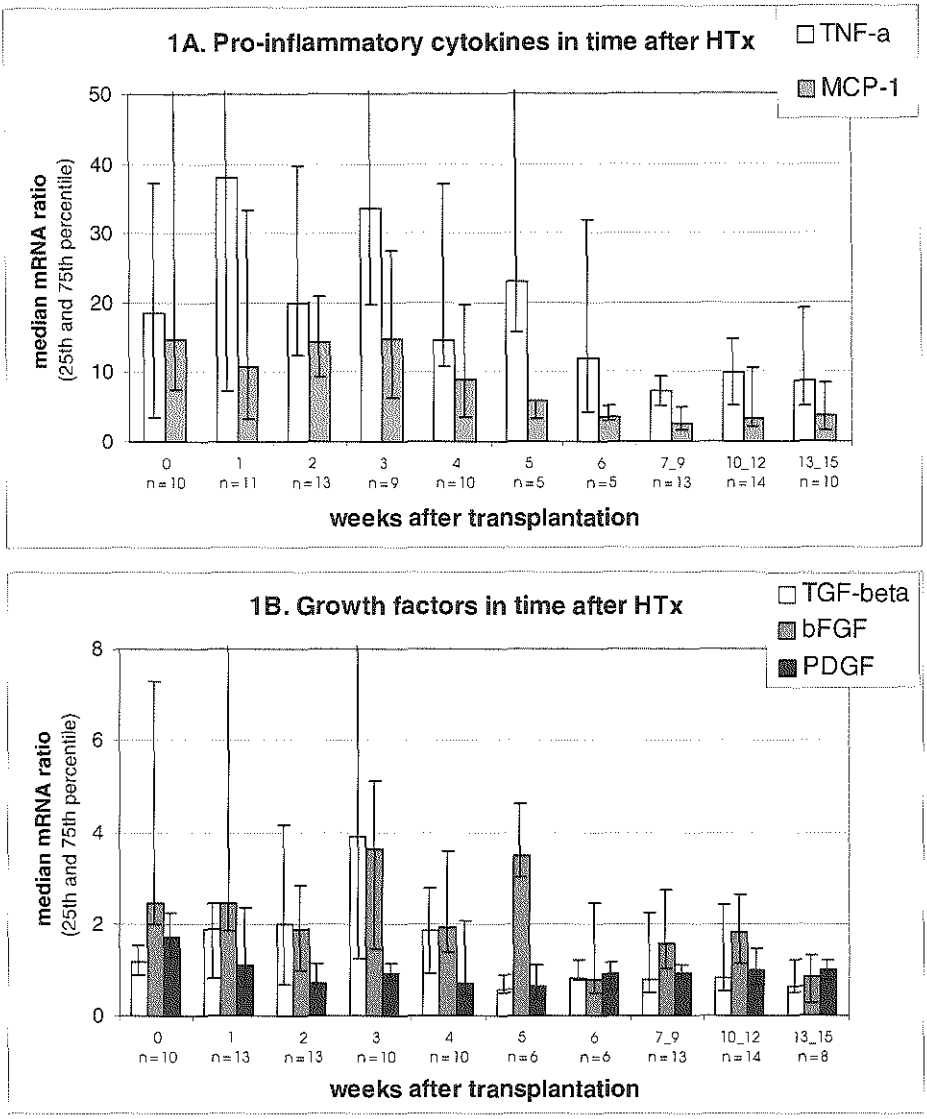

Figure 1: *Median cytokine/GAPDH mRNA expression levels of pro-inflammatory cytokines (1A), and growth factors (1B), during follow up after heart transplantation (acute rejection episodes excluded).*

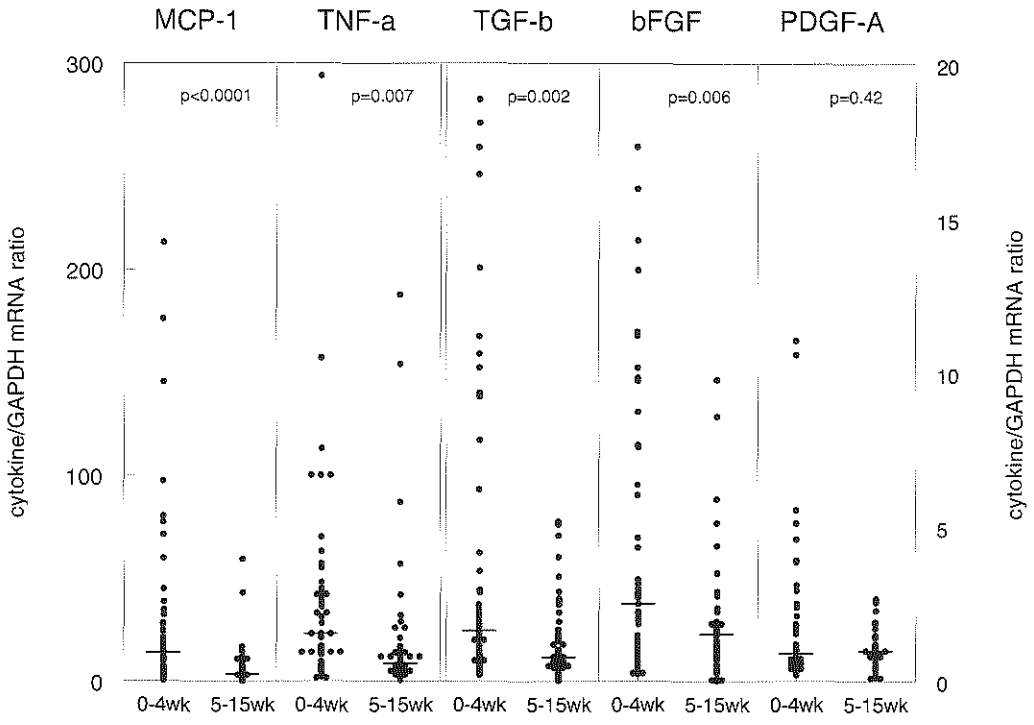

Figure 2: *Cytokine/GAPDH mRNA expression levels in the first month (0-4weeks) posttransplant versus the second/third month (5-15 weeks) posttransplant.*

Length of the duration of cold ischemia ranged from 112 to 272 minutes, and was not correlated with cytokine mRNA expression levels, neither in the first EMB (TNF-α: r = -0.16, p = 0.61; MCP-1: r = -0.20, p = 0.53; TGF-β: r = -0.19, p = 0.49; PDGF-A: r = -0.02, p = 0.95; bFGF: r = -0.09, p = 0.76) nor with median ratios during the first month post-transplant (TNF-α: r = -0.15, p = 0.59; MCP-1: r = -0.23, p = 0.39; TGF-β: r = -0.01, p = 0.98; PDGF-A: r = 0.16, p = 0.56; bFGF: r = 0.01, p = 0.98).

In four of sixteen patients no histological signs of acute rejection were observed during the first three months post-transplant. The remaining twelve patients had one or more biopsies with histological signs of acute rejection. Overall, histological signs of acute rejection were found in 20 of 123 biopsies. Most episodes of acute rejection occurred between the fourth and the sixth postoperative week. We compared the cytokine mRNA expression levels in all biopsies with and without acute rejection. This revealed

that acute rejection was not associated with the mRNA expression levels of MCP-1, TGF-β, PDGF-A, or bFGF. In contrast, Figure 3 shows that the expression level of TNF-α mRNA was significantly higher in the biopsies sampled during rejection as compared to non-rejection biopsies (p = 0.008).

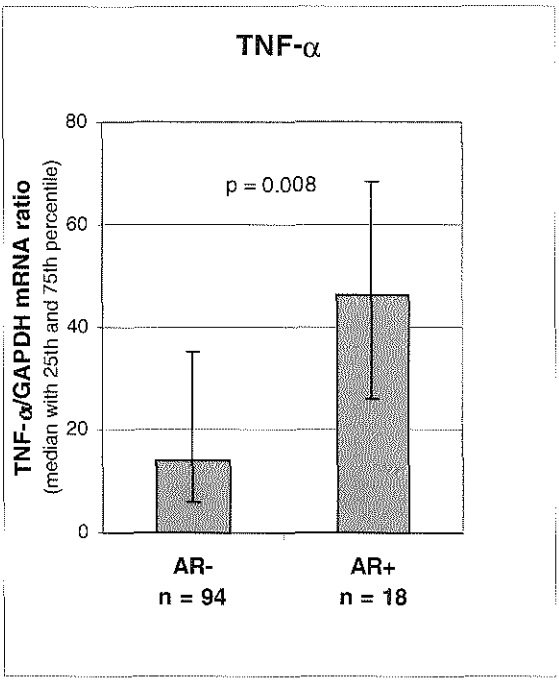

Figure 3: *Median TNF-α/GAPDH mRNA expression levels in biopsies without (AR-) and with (AR+) histological signs of acute rejection after heart transplantation.*

Next, we analysed median cytokine mRNA expression levels before, during, and after individual rejection episodes in the 12 rejecting patients to identify cytokine patterns around individual rejection episodes (Figure 4). We observed a trend towards higher median TNF-α mRNA expression during individual rejection episodes. Median MCP-1 mRNA expression was higher before and during rejection compared to the biopsy after rejection. In the first biopsy after treatment of acute rejection decreased median TGF-β mRNA expression levels as well as increased PDGF-A and bFGF mRNA expression levels were found. However, these associations were not statistically significant.

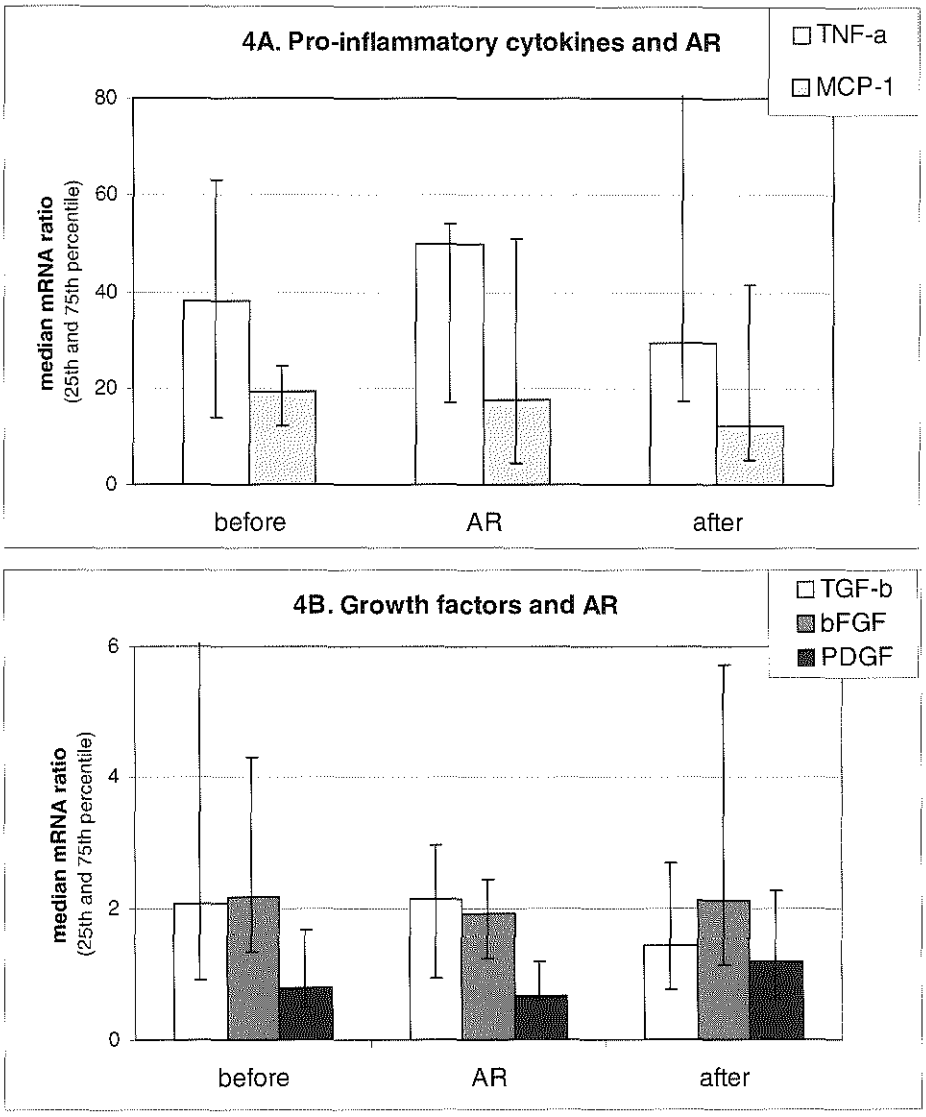

Figure 4: *Median cytokine/GAPDH mRNA expression levels of pro-inflammatory cytokines (4A), and growth factors (4B) in biopsies sampled before, during and after histological signs of acute rejection in heart transplant recipients.*

Discussion

In the present study we found a relatively high mRNA expression level of TNF-α, MCP-1, TGF-β and bFGF early after transplantation. These findings are in line with previous reports of elevated cytokine levels in serum (TNF-α, IL-6, IL-8) early after clinical heart

transplantation.[15,16] The increased intragraft cytokine expression in the first postoperative month most likely reflects the inflammatory response triggered by surgery, ischemia, and reperfusion, leading to cell activation and/or tissue injury.[3,11,17] However, we could not confirm a direct relationship between ischemic time, and cytokine mRNA expression levels in the graft, suggesting that ischemia is not the major factor leading to a cytokine response early after cardiac transplantation. The fact that ischemic times after heart transplantation are relatively short compared to other transplanted organs (generally within 2-4 hours) might be another explanation for the lack of association between ischemic times and cytokine mRNA expression levels in our study.

When we excluded acute rejection episodes, we showed a significant decrease in the measured cytokine mRNA expression levels after the first postoperative month (Figure 1 and 2). This is probably due to down-regulation of the early non-specific inflammatory response. The transcription factor NF-kB might play a central role in this process, because it enhances the gene expression of many pro-inflammatory mediators including cytokines.[18,19] It is likely that early after transplantation the production of NF-kB is upregulated by ischemia and reperfusion.[19] Because it is known that cyclosporine interferes with the activation of NF-kB[18], the decreased cytokine mRNA levels in the graft after the first month posttransplant might also be the effect of the ongoing immunosuppressive therapy.

During acute rejection we found higher expression levels of TNF-α mRNA within the graft. This is in agreement with previous observations that TNF-α mRNA and protein expression as well as TNF-α gene polymorphism were associated with acute allograft rejection.[3,11,20] Furthermore, TNF-α mRNA and protein expression in "time zero" biopsies have been associated with right ventricular failure after clinical heart transplantation.[21] TNF-α in the heart is produced by cardiac myocytes and macrophages, and is able to stimulate vascular endothelial cells to express adhesion molecules (eg VCAM-1) and HLA. This triggers increased adherence of monocytes and T cells to the endothelium, followed by infiltration into the cardiac tissue.[3,11] Our data underline the important role of this pro-inflammatory cytokine in the regulation of the allo-immune response after clinical heart transplantation.

Previous serial measurements of cytokines in serum or in the graft could not predict acute allograft rejection.[7,9,15,16] Our intragraft measurements also failed to identify an

individual predictive parameter for development of acute rejection. We cannot entirely exclude the influence of bacterial or viral infection episodes such as CMV on cytokine mRNA expression within the graft[22], although intragraft cytokine measurements do not automatically reflect peripheral events. After individual acute rejection episodes we found upregulation of PDGF-A and bFGF mRNA expression. Other investigators have previously shown increased expression of PDGF-A and bFGF mRNA and protein in transplanted heart tissue compared with control hearts that was not associated with histological signs of acute rejection.[23,24,25] This may suggest that these growth factors play an important role in allo-antigen independent repair of tissue injury rather than in the allo-antigen dependent immune response.

In conclusion, the association between intragraft TNF-α mRNA expression and acute rejection episodes emphasizes the importance of this cytokine in allogeneic responses. Furthermore, the increased growth factor expression levels in allografts early after transplantation and after acute rejection indicate the activation of a repair response to restore tissue injury caused by the transplantation procedure or by acute rejection episodes.

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CHAPTER 5

SEQUENTIAL MONITORING OF INTRAGRAFT CYTOKINE mRNA EXPRESSION IN RELATION TO DIASTOLIC LEFT VENTRICULAR WALL THICKNESS AND FUNCTION EARLY AFTER HEART TRANSPLANTATION

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Clinical Transplantation; in press.

Abstract

Because production of immune regulatory proteins may play a role in early graft dysfunction after heart transplantation, we analysed whether intragraft cytokine mRNA expression levels are associated with diastolic left ventricular function in cardiac allografts. We intensively monitored 16 cardiac allograft recipients during the first three months after transplantation. The messenger RNA (mRNA) expression levels of tumor necrosis factor-α (TNF-α), monocyte chemoattractant protein-1 (MCP-1), transforming growth factor-β (TGF-β), platelet derived growth factor-A (PDGF-A), and basic fibroblast growth factor (bFGF) were measured in endomyocardial biopsies (n = 123) by quantitative RT-PCR. To determine diastolic allograft function, concurrent M-mode and two-dimensional Doppler echocardiograms were analysed for the following parameters: left ventricular total wall thickness, maximal early and atrial mitral flow velocity, deceleration time of maximal early mitral flow velocity, and isovolumetric relaxation period. During the first 3 months post-transplant an overall decrease in mRNA expression levels of almost all measured cytokines was observed, which paralleled an improvement in diastolic left ventricular wall thickness and function. However, no straightforward relationship could be found between a specific cytokine mRNA expression pattern and the studied echocardiographic parameters. Our data suggest that the improvement in diastolic left ventricular function is associated with a general reduction of inflammation within the allograft, rather than related to a specific cytokine expression pattern.

Introduction

Acute rejection and diastolic dysfunction are major problems early after clinical heart transplantation.[1,2] Acute rejection can be the direct cause for impaired cardiac allograft function, but diastolic dysfunction is not always the result of an acute rejection episode.[3] For example, Ross et al. demonstrated an association between early diastolic dysfunction and reduced survival after transplantation, independent of histological evidence of rejection as characterized by mononuclear cell infiltration and myocyte injury.[2] This observation supports the hypothesis that tissue injury is not the only mechanism for impaired graft function. Instead, a number of investigators have suggested a role for cytokines in the pathophysiology of cardiac allograft

dysfunction.[1,3,4] Cytokines are regulatory proteins that play a central role in the anti-donor immune response. Their release by vascular endothelial cells, cardiac myocytes or inflammatory cells, can be triggered by various allo-antigen dependent and independent factors.[5,6]

The mechanism by which cytokines can influence cardiac function may be through interfering with cyclicAMP metabolism in myocytes and thus uncoupling the β-adrenoceptor from its second messenger system, or through promoting NO overproduction which has been shown to inhibit adrenergic stimulation of myocyte contractility.[4] Indeed, in experimental studies a variety of cytokines, including interleukin (IL)-1, IL-2, IL-6, tumor necrosis factor-α (TNF-α), and basic fibroblast growth factor (bFGF), have been shown to reversibly inhibit contractility of cardiac myocytes after β-adrenergic stimulation.[4] However, the relation between cytokines and diastolic dysfunction in transplant recipients has only been studied in a limited way.[7-9] In most of these studies cytokine expression was measured in serum or plasma, which does not necessarily reflect the cytokine response within the graft.[10,11] Furthermore, cytokines and graft function were studied at a single time point, thereby ignoring the influence of time after transplantation and/or rejection episodes on both parameters.

The aim of the present study was to analyse patterns of intragraft cytokine messenger RNA (mRNA) expression and echocardiographic parameters in time and close to histological rejection episodes during the first months after clinical heart transplantation, and subsequently correlate intragraft cytokine mRNA expression levels with echocardiographic parameters. Therefore, we measured a broad range of cytokines, which can be released by activated endothelial cells, cardiac myocytes and/or infiltrated mononuclear cells: TNF-α, monocyte chemoattractant protein-1 (MCP-1), transforming growth factor-β (TGF-β), platelet derived growth factor (PDGF-A), and bFGF. TNF-α and MCP-1 play a significant role in the regulation and recruitment of cells that participate in inflammatory responses, while the growth factors TGF-β, PDGF-A and bFGF are potent mitogenic proteins for fibroblasts, smooth muscle cells and endothelial cells, which is important in tissue repair processes. Echocardiographic measurements were focused on diastolic left ventricular wall thickness and function: left ventricular total wall thickness (TWT), maximal early (E) and atrial (A) mitral flow velocities, deceleration time of E (DET), and isovolumetric relaxation period (IRP).

Table 1: *Demographics of the studied patients, and number of endomyocardial biopsies (EMB) positive for acute rejection (AR+).*

	age	*gender*	*primary disease*	*ischemic time (min)*	*induction therapy with h-ATG*	*number of studied EMB*	*number of AR+ EMB*
1	20	F	CMP	182	no	8	2
2	52	M	IHD	166	yes	9	0
3	60	M	IHD	272	yes	excluded from the study	
4	48	M	IHD	152	no	9	2
5	58	M	CMP	134	yes	8	1
6	51	M	CMP	174	yes	8	1
7	56	F	CMP	127	no	7	1
8	58	M	IHD	173	no	9	2
9	53	F	CMP	147	no	8	4
10	50	M	IHD	157	no	7	1
11	16	F	CMP	189	yes	6	2
12	54	M	IHD	200	no	8	2
13	43	M	CMP	218	no	8	2
14	55	M	IHD	157	yes	9	0
15	58	M	IHD	247	no	9	0
16	52	M	IHD	112	no	10	0
						123	20

F: female, M: male, CMP: cardiomyopathy, IHD: ischemic heart disease, h-ATG: horse-antithymocyte globulin, EMB: endomyocardial biopsy, EMB AR+: endomyocardial biopsy showing acute rejection.

Patients and methods

Patients

We studied 16 consecutive heart transplant recipients who were operated upon between November 1997 and October 1998. The demographics of these patients are summarized in Table 1. Six of 16 patients received induction therapy for 5-7 days with horse-antithymocyte globulin (h-ATG; Institute Merieux, Lyon, France). Maintenance immunosuppressive therapy consisted of cyclosporin A and low dose steroids without addition of azathioprine or mycophololate mofetil. Serial endomyocardial biopsies (EMB) and concurrent echocardiograms were studied during the first 3 months post-transplant. Timing of surveillance biopsies in this period was weekly during the first 6 weeks and biweekly during the following 8 weeks. During routine biopsy sampling, an additional biopsy was harvested for cytokine studies after informed consent of the patients. One patient had been excluded from our analysis because of the poor quality

of serial echocardiograms. In total, 123 biopsies with concurrent echocardiograms (on average eight time points per patient) were available for analysis. Acute rejection was diagnosed by histological assessment of myocardial biopsies and graded according to the guidelines of the International Society for Heart and Lung Transplantation (ISHLT). [12] Patients with ISHLT rejection grade >2 were considered to have an acute rejection (AR+) and received additional immunosuppressive treatment. In four of 16 patients no histological signs of acute rejection were observed during the first 3 months post-transplant. The remaining 12 patients had one or more biopsies with histological signs of acute rejection. Overall, histological signs of acute rejection were found in 20 of 123 biopsies (Table 1). Most episodes of acute rejection occurred between the fourth and the sixth postoperative week.

Cytokine mRNA detection by quantitative RT-PCR

Competitive reverse transcriptase polymerase chain reaction (RT-PCR) was used for quantitative measurement of the following cytokines: TNF-α, MCP-1, TGF-β, PDGF-A, bFGF, and the constitutively expressed housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Total RNA was extracted from snapfrozen EMB and subsequently cDNA was synthesized with random primers as described previously in detail.[13] Aliquots of cDNA (representing 1/20 EMB) were directly used for PCR amplification, using sequence-specific primers for TNF-α (sense: 5'-GAG-TGA-CAA-GCC-TGT-AGC-CCA-TGT-TGT-AGC-A-3', antisense: 5'-GCA-ATG-ATC-CCA-AAG-TAG-ACC-TGC-CCA-GAC-T-3'), MCP-1 (sense: 5'-TAG-CAG-CCA-CCT-TCA-TTC-C-3', anti-sense: 5'-TTC-CCC-AAG-TCT-CTG-TAT-CT-3'), TGF-β (sense: 5'-GCC-CTG-GAC-ACC-AAC-TAT-TGC-3'; anti-sense: 5'-GCT-GCA-CTT-GCA-GGA-GCG-CAC-3'), PDGF-A (sense: 5'-AGA-AGT-CCA-GGT-GAG-GTT-AGA-GGA-GCA-T-3'; anti-sense: 5'-CTG-CTT-CAC-CGA-GTG-CTA-CAA-TAC-TTG-CT-3'), bFGF (sense: 5'-GGC-TTC-TTC-CTG-CGC-ATC-CA-3', anti-sense: 5'-GCT-CTT-AGC-AGA-CAT-TGG-AAG-A-3'), and GAPDH (sense: 5'-GGT-GAA-GGT-CGG-AGT-CAA-CG-3'; anti-sense: 5'-CAA-AGT-TGT-CAT-GGA-TGA-CC-3'). EMB negative for GAPDH mRNA expression were excluded from further analysis.

To estimate the relative initial amount of functional cytokine mRNA in EMB, a competitive template RT-PCR assay was used and normalized against GAPDH. Known

amounts of internal control fragment in different dilutions were added to constant amounts of sample cDNA for competitive co-amplification. We designed the internal controls to generate a smaller PCR product to allow differentiation between the amplified target and the internal control. Sample cDNA and internal control, 5 μl each, were added to a 90-μl PCR mixture containing 10 mM Tris-HCl, pH 8.3; 50 mM KCl; 1.5 mM $MgCl_2$; 0.2 mM of each dATP, dCTP, dTTP, dGTP; and 2 U Ampli Taq Gold (PE Biosystems, Norwalk CT, USA) overlaid with 100 μl mineral oil (Sigma, St Louis MO, USA) before PCR reaction in a DNA thermal cycler model 480 (PE Biosystems). After 10-min 94°C denaturation, samples underwent 40 cycles of 1-min denaturation at 94°C; 2-min annealing at optimal temperatures for TNF-α (60°C), MCP-1 (56°C), TGF-β (60°C), PDGF-A (60°C), bFGF (58°C), or GAPDH (60°C); and 3-min extension at 72°C. The last cycle was extended 7 min at 72°C. Positive control samples were produced by mRNA extraction and cDNA synthesis from 10^6 human spleen cells stimulated with 1% phytohemagglutin-M (PHA; Difco, Detroit MI, USA) for 24 hours at 37°C. Negative control samples consisted of diethyl pyrocarbonate-treated H_2O as no-template reaction. The relative intensity of internal control and target products on gel was measured by luminescence with a DC-40 camera in combination with analysis software (Kodak, Rochester NY, USA). Subsequently, the relative concentration of cytokine mRNA was divided by the relative concentration of GAPDH mRNA in the same sample, to indicate the initial cytokine mRNA expression level in EMB.

Echocardiography

Echocardiographic examination was completed within 4 hours of biopsy sampling using a Hewlett Packard Sonos 5500 ultrasonograph with a 3.75 MHz transducer (Hewlett Packard, Andover, MA, USA). All recordings were performed and analysed by a single investigator without knowledge of the results of endomyocardial biopsies. M-mode recordings, for measurement of left ventricular wall dimensions, were obtained from the parasternal long axis view in combination with an electrocardiogram, phonocardiogram and respiratory tracing. For two-dimensional Doppler echocardiography the transducer was positioned at the cardiac apex for a standard apical four-chamber view. Mitral flow velocities were recorded within the valve orifice near the leaflet tips with the sample volume placed parallel to the ventricular inflow tract.

Offline, M mode and two dimensional Doppler echocardiograms were analysed using a software program developed in our laboratory with a hand-held digitizer connected to a digitizing tablet (Summa Sketch Plus, Summagraphics, Seymour, USA) interfaced with a personal computer. For M-mode echocardiogram analysis, mean values of five separate consecutive end-expiratory beats were calculated. M-mode echocardiograms were analysed for end-diastolic posterior left ventricular wall thickness (PW) and end-diastolic interventricular septum thickness (IVS). Left ventricular total wall thickness (TWT) was calculated by adding end-diastolic posterior wall thickness and end-diastolic interventricular septum thickness. Two dimensional Doppler echocardiograms were analysed for the following diastolic function parameters: Peak early mitral flow velocity (E), peak atrial mitral flow velocity (A), E/A ratio, deceleration time of E (DET), and isovolumetric relaxation period (IRP). For Doppler cardiogram analysis, mean values of 10 consecutive end-expiratory beats were calculated. Beats that were distorted by the recipient atrial contraction were not analysed with respect to DET. The transmitral filling pattern was classified as "summation filling" when only one peak occurred in diastole after the P wave of the succeeding beat. In such recordings, E and A waves could not be recognized and consequently the parameters E, A, E/A ratio and DET could not be measured.

Statistics

Changes in echocardiographic parameters in time were determined by one-way analysis of variance (ANOVA). Differences in median cytokine mRNA expression levels between EMB sampled during the first month and during the second/third month post-transplant were analysed by non-parametric Mann-Whitney *U*-test. Differences in mean echocardiographic parameters between EMB with and without histologically proven acute rejection were analysed by Student's *t*-test. Correlations between cytokine mRNA expression levels and echocardiographic variables, as well as correlations of the duration of cold ischemia with both parameters, were defined by Pearson's correlation coefficient (r) after log-transformation of the cytokine mRNA ratios. Associations with $p < 0.05$ were considered statistically significant.

Results

Cytokine mRNA expression

To determine the relationship between cytokine mRNA expression levels and time after transplantation we excluded the biopsies sampled during, and 3 weeks following an acute rejection episode. Figure 1 shows that the expression levels of MCP-1, TNF-α, bFGF, and TGF-β mRNA were significantly higher in the first post-operative month compared with the period thereafter. Median cytokine/GAPDH mRNA ratio in the first month vs. 2-3 months post-transplant were 13.8 vs. 3.4 for MCP-1 ($p < 0.0001$), 23.0 vs. 8.6 for TNF-α ($p = 0.007$), 25.0 vs. 15.2 for bFGF ($p = 0.006$), and 16.4 vs. 7.8 for TGF-β ($p = 0.002$).

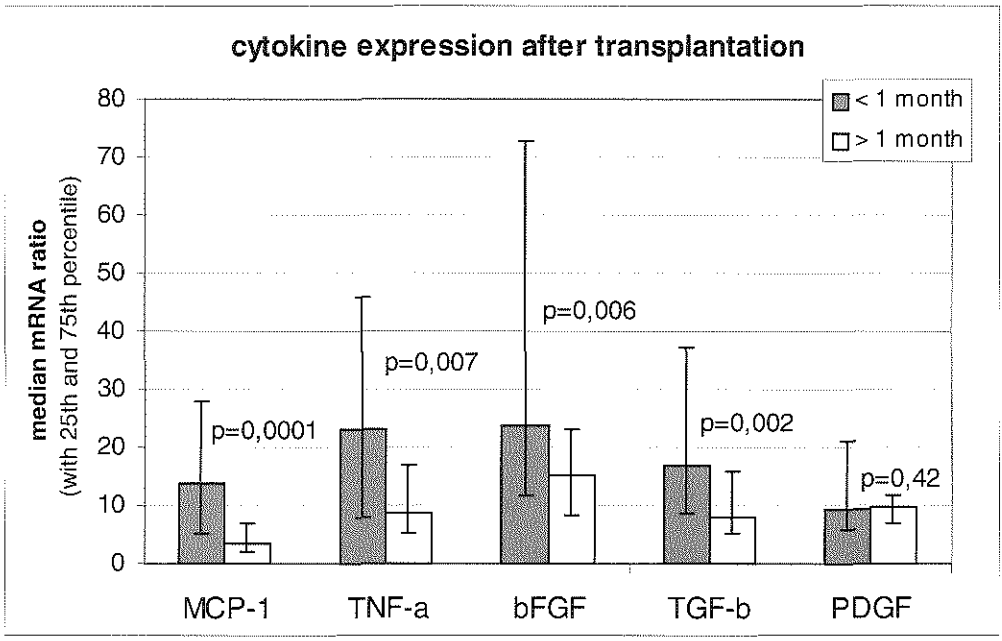

Figure 1: *Median cytokine mRNA expression levels in the first month compared to the second/third month posttransplant, acute rejection episodes excluded.*

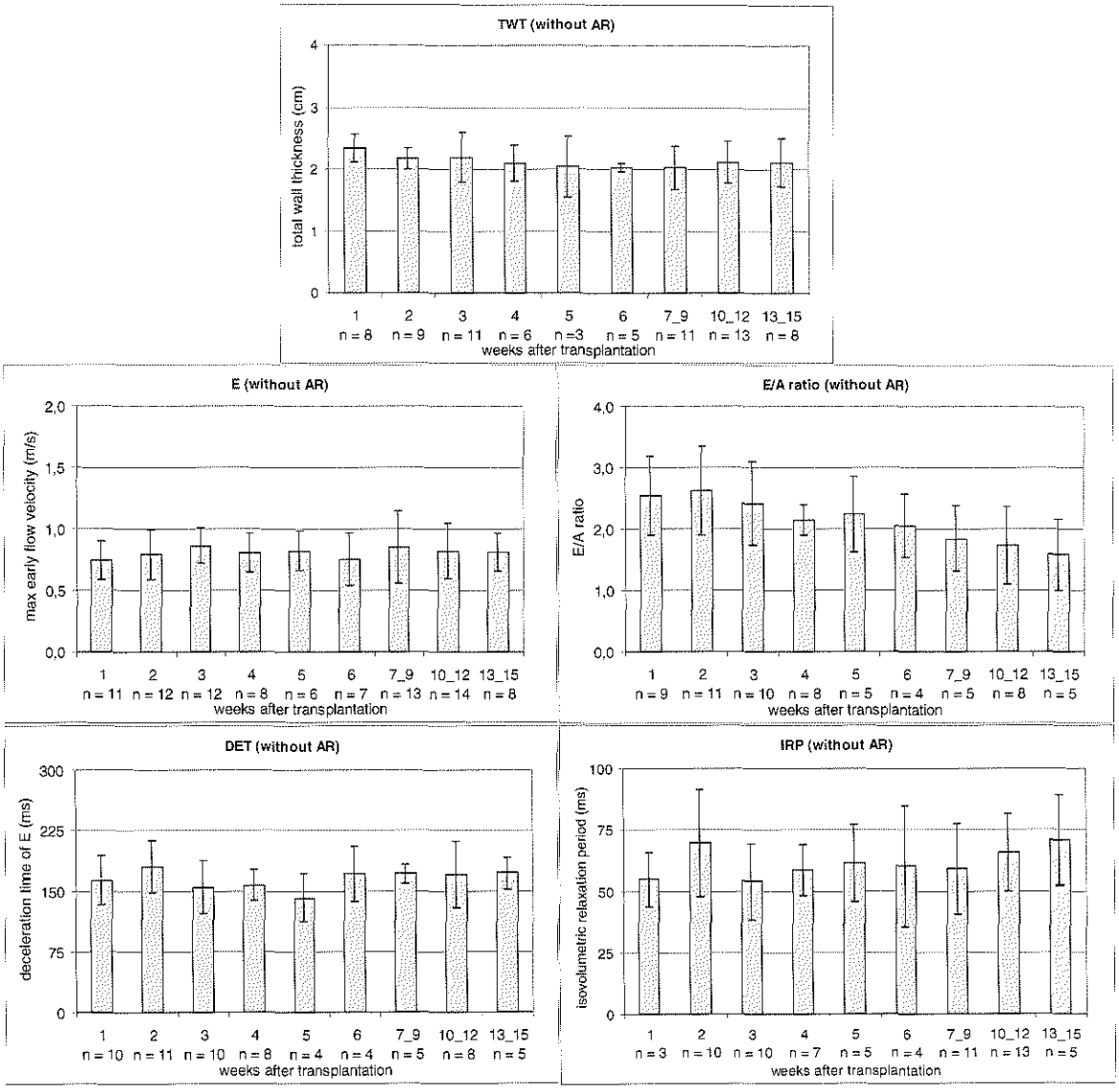

Figure 2: *Mean echocardiographic parameters (with standard deviations) during follow up, acute rejection episodes excluded. The E/A ratio decreased significantly during the three month study period (p = 0.014).*

Length of the duration of cold ischemia (Table 1) was not correlated with cytokine mRNA expression levels, neither in the first EMB (TNF-α: r = -0.16, p = 0.61; MCP-1: r = -0.20, p = 0.53; TGF-β: r = -0.19, p = 0.49; PDGF-A: r = -0.02, p = 0.95; bFGF: r

= -0.09, p = 0.76) nor with median ratios during the first month post-transplant (TNF-α: r = -0.15, p = 0.59; MCP-1: r = -0.23, p = 0.39; TGF-β: r = -0.01, p = 0.98; PDGF-A: r = 0.16, p = 0.56; bFGF: r = 0.01, p = 0.98).

Diastolic wall thickness and function

We observed a large individual variation in the echocardiographic measurements. First, we determined the influence of time separately from the influence of acute rejection by excluding the measurements during histological signs of rejection and during treatment/recovery after rejection (3 weeks following the last rejection biopsy). Figure 2 shows the mean value of the different echocardiographic parameters from all patients during serial measurements. These data revealed a tendency toward improved function in the first three post-operative months: a decrease in TWT, an increase in IRP, and a significant decrease in E/A ratio (p = 0.014). No correlation was found between duration of cold ischemia times (Table 1) and any of the echocardiographic parameters neither as measured during the first week (TWT: r = -0.32, p = 0.34; E: r = 0.17, p = 0.58; E/A ratio: r = -0.22, p = 0.54; DET: r = 0.50, p = 0.12; IRP: r = 0.13, p = 0.87) nor as the mean of all measurements during the first month (TWT: r = -0.39, p = 0.16; E: r = 0.29, p = 0.28; E/A ratio: r = 0.10, p = 0.73; DET: r = -0.05, p = 0.87; IRP: r = -0.07, p = 0.80).

Analysis of diastolic left ventricular wall thickness and function before, during, and after acute rejection episodes, revealed no significant differences in echocardiographic variables (Figure 3). To standardize for intra-individual variation in echocardiographic parameters, we also measured percentage change toward and after individual acute rejection episodes. In this way, each patient served as its own control. However, histological signs of acute rejection were again not associated with individual changes in any of the studied diastolic echocardiographic parameters (data not shown). Systolic function in this patient group remained overall normal, but the quality of the measurements of left ventricular ejection fraction did not allow calculation of changes.

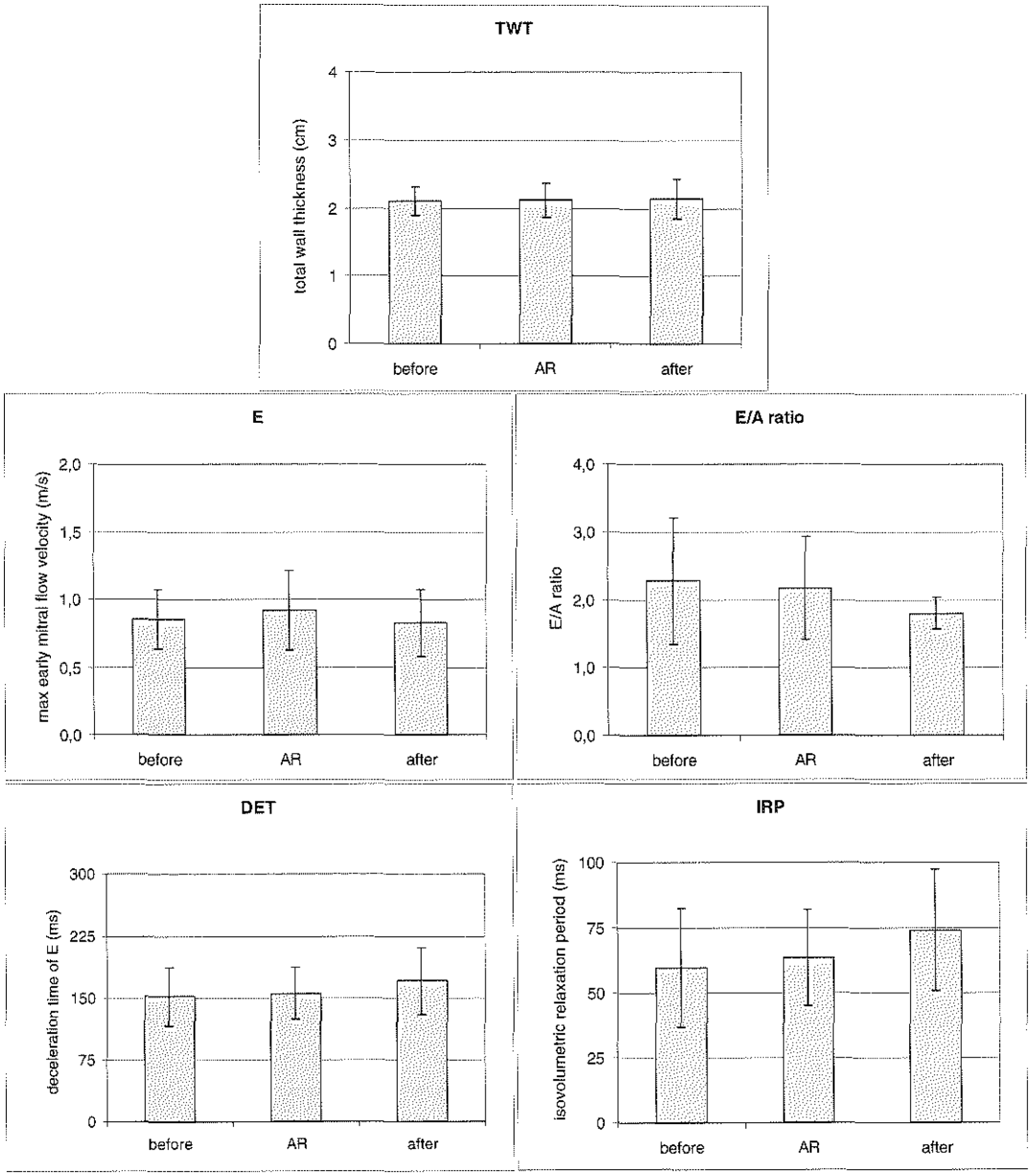

Figure 3: *Mean echocardiographic variables (with standard deviations) at the timepoint of biopsies sampled before, during, and after histological signs of acute rejection (AR).*

Table 2a: *Correlation between cytokine/GAPDH mRNA ratio and echocardiographic variables as measured at the same day. * $p < 0.05$.*

	TNF-α	*MCP-1*	*TGF-β*	*PDGF-A*	*bFGF*
TWT	r = 0.10	r = 0.23	r = 0.01	r = 0.003	r = 0.19
	p = 0.34	p = 0.02 *	p = 0.91	p = 0.97	p = 0.06
E	r = 0.16	r = -0.07	r = 0.05	r = -0.71	r = -0.09
	p = 0.10	p = 0.47	p = 0.55	p = 0.44	p = 0.31
E/A ratio	r = -0.16	r = -0.18	r = 0.04	r = -0.22	r = -0.12
	p = 0.17	p = 0.10	p = 0.74	p = 0.04 *	p = 0.29
DET	r = -0.07	r = -0.01	r = -0.22	r = 0.03	r = -0.03
	p = 0.56	p = 0.92	p = 0.04 *	p = 0.81	p = 0.80
IRP	r = 0.35	r = 0.28	r = 0.03	r = -0.01	r = 0.12
	p = 0.001 *	p = 0.005 *	p = 0.79	p = 0.95	p = 0.25

E: peak early mitral flow velocity, A: peak atrial flow velocity, DET: deceleration time of E, IRP: isovolumetric relaxation period.

Table 2b: *Correlation between cytokine/GAPDH mRNA ratio and percentual change in echocardiographic variables toward the subsequent measurement. * $p < 0.05$.*

	TNF-α	*MCP-1*	*TGF-β*	*PDGF-A*	*bFGF*
ΔTWT	r = 0.05	r = 0.08	r = 0.08	r = -0.08	r = 0.01
	p = 0.64	p = 0.45	p = 0.47	p = 0.48	p = 0.91
ΔE	r = 0.11	r = 0.10	r = 0.11	r = 0.19	r = 0.11
	p = 0.29	p = 0.29	p = 0.26	p = 0.06	p = 0.25
ΔE/A ratio	r = 0.17	r = 0.14	r = 0.05	r = 0.06	r = 0.19
	p = 0.20	p = 0.28	p = 0.72	p = 0.63	p = 0.13
ΔDET	r = -0.21	r = -0.09	r = -0.05	r = -0.13	r = -0.16
	p = 0.11	p = 0.46	p = 0.69	p = 0.31	p = 0.21
ΔIRP	r = -0.20	r = -0.03	r = 0.06	r = 0.04	r = 0.01
	p = 0.09	p = 0.81	p = 0.61	p = 0.74	p = 0.91

E: peak early mitral flow velocity, A: peak atrial flow velocity, DET: deceleration time of E, IRP: isovolumetric relaxation period.

Cytokine expression and allograft function

To determine the influence of individual intragraft cytokine expression on diastolic left ventricular wall thickness and function, we calculated the correlation between cytokine mRNA expression levels in all biopsies and echocardiographic parameters (Table 2a and b). Although a few combinations as measured at the same day were positively or negatively correlated (Table 2a), the data show a diffuse distribution and do not reveal a consistent picture for the relation between a specific cytokine mRNA expression pattern and cardiac function. To standardize for individual differences in echocardiographic parameters, we subsequently calculated the percentage change between echocardiographic variables at each timepoint of cytokine measurement and the subsequent echocardiographic measurement. However, none of the cytokine expression levels were significantly correlated with individual improvement or deterioration in allograft function toward the subsequent measurement (Table 2b).

Discussion

Although the histological grading system after heart transplantation is generally indicative of clinical relevant rejection, unexplained episodes of cardiac allograft dysfunction do occur.[1-3] Because such episodes unaccompanied by histological evidence of significant cellular rejection may improve with enhanced immunosuppression therapy, it has been speculated that left ventricular dysfunction after heart transplantation is not only dependent on the presence and cytotoxic effects of infiltrated immune cells, but also on the production of cytokines by endothelial cells, cardiac myocytes or infiltrating cells.[1,4]

In the present study we found relatively high mRNA expression levels of TNF-α, MCP-1, TGF-β and bFGF early after transplantation, which decreased after the first post-operative month. This may be the result of the activation of a (non-specific) inflammatory response triggered by donor brain death, surgery, ischemia, and reperfusion, leading to cell activation and tissue injury.[5,14] Our findings are in line with previous reports of elevated cytokine mRNA expression levels (IL-2, IL-4 and TNF-α) in the graft early after heart transplantation.[10,11] Furthermore, our results showed an improvement of the measured left ventricular function parameters during the first months post-transplant. This has also been observed by other investigators and has been

ascribed to recovery from the operative insult associated with ischemia, raised catecholamine concentrations and increased filling pressures.[15,16] There was, however, no evidence for a direct relationship between the duration of cold ischemia and cytokine mRNA expression levels or echocardiographic parameters, suggesting that ischemia is not the only factor leading to a cytokine response and graft dysfunction early after heart transplantation.

Conflicting data on the association between cardiac allograft function and acute rejection have been published.[17-21] Although some authors described an association between acute rejection and echocardiographic parameters at group level, the considerable overlap between individuals limits the use of echocardiography for the diagnosis of acute rejection. In the present study, no correlation has been found between any of the echocardiographic variables and histological signs of acute allograft rejection. Because variation in pre- and afterload are important determinants of the transmitral flow properties, this lack of correlation can, besides the risk of false negative biopsy samples, be explained by changes in loading conditions caused by post-transplant arterial hypertension, or by fluid retention as a result of the use of cyclosporine and prednisone.[21,22] Furthermore, the above mentioned gradual improvement in diastolic function in the early post-operative period may conceal acute changes caused by the rejection process.

A variety of cytokines (IL-1, IL-2, IL-6, IL-8, TNF-α, IFN-γ, and bFGF) has been shown to influence the function of cardiac myocytes in experimental and in clinical studies.[23,24] In patients undergoing coronary bypass grafting, an association has been reported between left ventricular wall motion abnormalities and increased plasma levels of IL-6 and IL-8.[25] Moreover, in a similar patient population, a low ejection fraction has been associated with higher pre-operative IL-2 and TNF-α serum levels.[26] In patients with chronic heart failure, increased circulating TNF-α levels have often been described.[27,28] Furthermore, increased intracardial expression of TNF-α mRNA and protein has been found in failing hearts from patients before transplantation, as compared to non-failing post-mortem hearts.[29] After heart transplantation, intragraft mRNA expression of IL-6, IL-10, TNF-α and TGF-β has been found in a higher proportion of heart transplant recipients with abnormal function at 6 weeks to 7 months post-transplant.[7] Elevated serum levels of IL-6 and TNF-α were reported to correlate with diastolic and systolic allograft dysfunction

early after heart transplantation in the absence of rejection.[8] In contrast, another study reported that IL-6 serum levels were not related with clinically relevant hemodynamic changes during cardiac allograft rejection.[9]

Although we showed that a decline in cytokine mRNA expression level was accompanied by improvement of diastolic left ventricular function in the first post-operative months, we did not find a straightforward correlation between specific cytokine mRNA expression patterns and changes in echocardiographic variables. This lack of correlation between intragraft cytokine levels and cardiac function parameters suggests that the improvement in diastolic left ventricular wall thickness and function depend on a decrease of inflammation in general, rather than on a specific intragraft cytokine expression pattern. The discrepancy between our results and previous studies may be because the cytokine concentrations used in experimental studies are higher than expression levels in vivo, and because in clinical studies different sources of patient material (intragraft or peripheral) and different methods for cytokine measurement are used. In addition, because cytokine expression has been more often related to systolic instead of diastolic cardiac function, the relation between cytokines and allograft function might be more pronounced when cardiac function has deteriorated further to systolic dysfunction.[26] Moreover, in clinical studies, the use of immunosuppressive drugs can influence cytokine mRNA expression as well as diastolic left ventricular wall thickness and function.[22]

To our knowledge, this is the first report correlating intragraft cytokine mRNA expression levels with diastolic left ventricular function parameters early after clinical heart transplantation in a serial study design. In summary, our results show a declined cytokine mRNA expression level as well as an improvement of cardiac allograft function during the first months after transplantation. However, no direct association has been found between intragraft cytokine mRNA expression levels and diastolic left ventricular function parameters. We conclude that early diastolic dysfunction after clinical heart transplantation is not directly related to a specific intragraft cytokine expression pattern.

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CHAPTER 6

INTRAGRAFT INTERLEUKIN-2 mRNA EXPRESSION DURING ACUTE CELLULAR REJECTION AND LEFT VENTRICULAR TOTAL WALL THICKNESS AFTER HEART TRANSPLANTATION

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Heart 2002; 87 (4): 363 - 367.

Abstract

The objective was to assess whether diastolic graft function is influenced by intragraft Interleukin-2 (IL-2) messenger RNA (mRNA) expression in rejecting cardiac allografts. Sixteen recipients of cardiac allografts were monitored during the first three months after transplantation. The presence of IL-2 mRNA in endomyocardial biopsies (EMB) (n = 123) was measured by reverse transcriptase polymerase chain reaction (RT-PCR). To determine heart function, concurrent M mode and two dimensional Doppler echocardiograms were analysed. Histological signs of acute rejection (ISHLT rejection grade > 2) were strongly associated with IL-2 mRNA expression (IL-2 mRNA was present in 12 out of 20 EMB (60%) with acute rejection and in 24 out of 103 EMB (23%) without acute rejection, p = 0.002). No significant relation was found between either histology or IL-2 mRNA expression alone, and the studied echocardiographic parameters. However, stratification of the echocardiographic data into those of patients with and without acute rejection showed that during acute rejection IL-2 mRNA expression was significantly associated with increased left ventricular total wall thickness (mean change in total wall thickness was +0.22 cm in patients with IL-2 mRNA expression versus -0.18 cm in patients without IL-2 mRNA expression, p = 0.048). We conclude that an increased left ventricular total wall thickness precedes IL-2 positive acute rejection after heart transplantation. Thus, cardiac allograft rejection accompanied by intragraft IL-2 mRNA expression may be indicative for more severe rejection episodes.

Introduction

Reversible diastolic dysfunction of cardiac allografts in the first post-operative months has been reported to correlate with reduced long-term survival.[1] This diastolic dysfunction can be the result of acute rejection, a major complication shortly after transplantation.[2] However, not all histologically proven acute rejection episodes result in changes of diastolic function parameters. The underlying mechanism of the difference between acute rejection episodes with and without impaired heart function is unknown but may involve the local or systemic release of cytokines.[2]

Acute cellular rejection is morphologically characterized by the presence of a mononuclear cell infiltrate and signs of myocyte damage on endomyocardial biopsies

(EMB).[3] The combination of infiltrated immune competent cells with interstitial and perivascular edema, vascular leakage of fibrin, and in the more severe cases diffuse myocyte necrosis can result in stiffness of the myocardium.[4] Consequently, the acute rejection process may change left ventricular wall dimensions and left ventricular filling parameters, reflecting the loss of diastolic heart function. Previously reported echocardiographic changes related to acute cellular rejection include increased left ventricular total wall thickness, increased peak early mitral flow velocity (E), decreased deceleration time, and shortened isovolumetric relaxation period.[5,6] Alternatively, during vascular (humoral) rejection, cells of the microvasculature instead of myocytes might be target of tissue injury, which has been associated with increased left ventricular mass and depressed myocyte function.[4,7]

In addition to cell infiltration and tissue damage, local production of cytokines (low molecular weight regulatory proteins) during rejection may also influence myocardial function.[8,9] One of the crucial cytokines in the modulation of the allo-immune response is interleukin-2 (IL-2), which enhances the proliferation and differentiation of specific T lymphocytes directed against the allograft.[10,11] In non-transplant settings, IL-2 has been shown to cause dysfunction of the heart both in experimental and in clinical studies.[12,13] A dose dependent negative inotropic effect of IL-2 was observed in isolated hamster papillary muscle preparations.[12] Moreover, intravenous administration of IL-2 in patients with cancer causes cardiovascular side effects including decreased ejection fraction, decreased blood pressure, increased heart rate, and fluid retention.[13] Therefore, we speculate that after transplantation the local presence of IL-2 may be related to allograft dysfunction by reflecting the severity of the immune response against the allograft and by its direct negative inotropic effect on the myocardium. To determine whether local IL-2 expression during histological rejection indeed affects diastolic graft function, we monitored serially sampled endomyocardial biopsies for the presence of IL-2 mRNA expression, and for histological signs of acute rejection during the first three months after heart transplantation. These findings were analysed in relation to echocardiographic changes in wall dimensions and diastolic flow parameters.

Methods

Patients

We studied 16 consecutive heart transplant recipients who were operated upon between November 1997 and October 1998. Table 1 summarises the demographic data of these patients. Maintenance immunosuppressive therapy consisted of cyclosporin A and low dose steroids. Serial EMB and echocardiograms during the first three months after transplantation were studied. Surveillance biopsies in this period were taken weekly during the first six weeks and biweekly during the following eight weeks. During routine biopsies two additional samples were harvested for cytokine studies after informed consent of the patients. One patient has been excluded from analysis because of the poor quality of serial echocardiograms, leaving 15 patients in the study group. In total, 123 biopsies with concurrent echocardiograms (on average eight time points per patient) were available for analysis. Acute rejection was diagnosed by histological assessment of myocardial biopsies and graded according to the guidelines of the International Society for Heart and Lung Transplantation (ISHLT).[3] Patients with ISHLT rejection grade >2 were considered to have an acute rejection (AR+) and received additional immunosuppressive treatment.

Echocardiography

Echocardiographic examination was completed within four hours of biopsy sampling using a Hewlett Packard Sonos 500 or 5500 ultrasonograph (Hewlett Packard, Palo Alta, California, USA) with a 3.75 MHz transducer. All recordings were made and analysed by a single investigator without knowledge of the results of EMB. M mode recordings for measurement of left ventricular wall dimensions were obtained from the parasternal long axis view in combination with an ECG, phonocardiogram and respiratory tracing. For two dimensional Doppler echocardiography the transducer was positioned at the cardiac apex for a standard apical four chamber view. Mitral flow velocities were recorded within the valve orifice near the leaflet tips with the sample volume placed parallel to the ventricular inflow tract.

Offline, M mode and two dimensional Doppler echocardiograms were analysed using a software program developed in our laboratory with a hand held digitizer connected to a digitizing tablet (Summa Sketch Plus, Summagraphics, Seymour, Connecticut, USA)

interfaced with a personal computer. For M mode echocardiogram analysis, means of five consecutive end expiratory beats were calculated. M mode echocardiograms were analysed for end diastolic posterior left ventricular wall thickness and end diastolic interventricular septum thickness. Left ventricular total wall thickness was calculated by adding end diastolic posterior wall thickness and end diastolic interventricular septum thickness. Two dimensional Doppler echocardiograms were analysed for the following diastolic function parameters: Peak early mitral flow velocity (E), peak atrial mitral flow velocity (A), E/A ratio, deceleration time of E, and isovolumetric relaxation period. For Doppler echocardiogram analysis, means of 10 consecutive end expiratory beats were calculated. Beats that were distorted by the recipient atrial contraction were not analysed with respect to deceleration time. The transmitral filling pattern was classified as "summation filling" when only one peak occurred in diastole after the P wave of the succeeding beat. In such recordings, E and A waves could not be recognized and consequently the parameters E, A, E/A ratio and deceleration time were not measured.

IL-2 mRNA detection

Reverse transcriptase polymerase chain reaction (RT-PCR) was used for detection of IL-2 mRNA expression. Therefore, Total RNA was extracted from snapfrozen EMB, and subsequently complementary DNA (cDNA) was synthesized with random primers as described previously in detail.[14] Aliquots of cDNA (representing 1/20 EMB) were directly used for PCR amplification using sequence-specific primers for IL-2 (sense: 5'-ATG.TAC.AGG.ATG.CAA.CTC.CTG.TCT.T-3', antisense: 5'-GTC.AGT.GTT.GAG.ATG.ATG.CTT.TGA.C-3'). Sample cDNA (5 μl) was added to a 95 μl PCR mixture containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dATP, dCTP, dTTP, dGTP, 2 U Taq-polymerase (Applied Biosystems, Norwalk, Connecticut, USA) and overlaid with 100 μl mineral oil (Sigma, St Louis, Missouri, USA) before PCR reaction in a DNA thermal cycler model 480 (Applied Biosystems) under the following conditions. After a 5 min 94°C denaturation step, samples underwent 40 cycles of 1 min denaturation at 94°C, 2 min annealing at 60°C, and 3 min extension at 72°C. The last cycle was extended with 7 min at 72°C.

Positive control samples were produced by messenger RNA extraction and cDNA synthesis from 10^6 random human spleen cells stimulated with 1% phytohemagglutin-M

(Difco, Detroit, Michigan, USA) for 24 hours at 37°C. Negative control samples consisted of diethyl pyrocarbonate-treated H_2O as no template reaction. In all samples, constitutively expressed glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was amplified in a separate PCR reaction (GAPDH primers: sense: 5'-GGT.GAA.GGT.CGG.AGT.CAA.CG-3'; anti-sense: 5'-CAA.AGT.TGT.CAT.GGA.TGA.CC-3') to confirm successful mRNA extraction and cDNA transcription. EMB that were negative for GAPDH mRNA expression were excluded from further analysis.

The presence of IL-2 mRNA on agarose gel was visually assessed as present (IL-2 positive) or not present (IL-2 negative) and confirmed by Southern blot hybridization. Therefore, products on agarose gel were transferred to a Hybond-N+ membrane (Amersham, Buckinghamshire, UK) by electroblotting and subsequently hybridized with a $\gamma^{32}P$ labeled specific probe for IL-2 (5'-TTC.TTC.TAG.ACA.CTG.AAG.ATG.TTT.CAG.TTC-3') which is located across the splice site. Hybridization was detected by autoradiography and indicated the presence of IL-2 mRNA expression in the original biopsy.

Statistics

Differences in mean echocardiographic parameters between EMB with and those without either histologically proven acute rejection or IL-2 mRNA expression were analysed by Student's *t* test. Associations between IL-2 mRNA expression and acute rejection, as well as changes in wall dimension between the first measurement and the first acute rejection biopsy with or without IL-2 mRNA expression, were calculated by Fisher's exact test in a cross table. Associations with $p < 0.05$ were considered significant.

Results

Acute rejection

In 4 of 16 patients no histological signs of acute rejection were observed during the first three months after transplantation. These non-rejectors served as control group. One of the rejectors (patients with at least one AR+ biopsy) was excluded from analysis because of poor echocardiographic quality. Overall, 20 of 123 EMB were positive for

acute rejection (16%). Table 1 shows the total number of biopsies with acute rejection during follow up of the patients.

Table 1: *Demographics of the studied patients and the numbers of endomyocardial biopsies (EMB) positive for acute rejection (AR+) and IL-2 mRNA expression (IL-2+).*

	age	*gender*	*primary disease*	*number of studied EMB*	*number of AR+ EMB*	*number of IL-2+ EMB*	*number of AR+/IL-2+*	*number of AR-/IL-2+*
1	20	F	CMP	8	2	1	1	0
2	52	M	IHD	9	0	5	0	5
3	60	M	IHD	excluded from the study due to poor echocardiographic quality				
4	48	M	IHD	9	2	4	2	2
5	58	M	CMP	8	1	1	1	0
6	51	M	CMP	8	1	2	1	1
7	56	F	CMP	7	1	3	1	2
8	58	M	IHD	9	2	3	2	1
9	53	F	CMP	8	4	2	0	2
10	50	M	IHD	7	1	1	0	1
11	16	F	CMP	6	2	2	2	0
12	54	M	IHD	8	2	4	1	3
13	43	M	CMP	8	2	3	1	2
14	55	M	IHD	9	0	2	0	2
15	58	M	IHD	9	0	2	0	2
16	52	M	IHD	10	0	1	0	1
				123	20	36	12	24

AR: acute rejection, CMP: cardiomyopathy, IHD: ischemic heart disease, IL-2: interleukin-2, F: female, M: male.

Table 2: *Cross-table of the number of biopsies negative and positive for acute rejection (AR-; AR+) and IL-2 mRNA expression (IL-2-; IL-2+).*

	AR-	*AR+*	*total*
IL-2-	79	8	87
IL-2+	24	12	36
total	103	20	123

IL-2 mRNA expression in relation to rejection

Serial measurements of IL-2 mRNA expression showed that every patient in the rejector-group and in the non-rejector-group had at least one IL-2 positive EMB during the first three months after transplantation. Overall, IL-2 mRNA expression was detected in 36 of 123 EMB (29%). Table 1 summarises the number of IL-2 positive EMB in the presence (AR+) or absence (AR-) of acute rejection during follow up. IL-2 mRNA expression was strongly associated with histological signs of acute rejection requiring treatment. IL-2 mRNA was present in 12 of 20 EMB (60%) with histological signs of acute rejection, and in 24 of 103 EMB (23%) without these histological characteristics (p = 0.002; Fischer's exact test, Table 2).

Table 3a: *Echocardiographic parameters at the time of EMB with (AR+) and without (AR-) acute rejection, and with (IL-2+) and without (IL-2-) IL-2 mRNA expression.*

	AR-	*AR+*	*p-value*	*IL-2-*	*IL-2+*	*p-value*
TWT cm, mean (SD)	2.12 (0.30)	2.12 (0.26)	1.00	2.15 (0.30)	2.12 (0.34)	0.65
E m/sec, mean (SD)	0.82 (0.22)	0.81 (0.23)	0.85	0.81 (0.20)	0.85 (0.24)	0.35
E/A ratio, mean (SD)	2.20 (0.70)	2.00 (0.78)	0.32	2.16 (0.74)	2.08 (0.61)	0.63
DET msec, mean (SD)	161.9 (36.6)	151.3 (29.8)	0.28	159.6 (30.2)	160.6 (45.0)	0.90
IRP msec, mean (SD)	64.83 (19.92)	69.4 (20.29)	0.41	64.41 (21.14)	66.73 (20.20)	0.63

A: peak atrial mitral flow velocity, AR: acute rejection, DET: deceleration time of E, E: peak early mitral flow velocity, EMB: endomyocardial biopsy, IL-2: interleukin-2, IRP: isovolumetric relaxation period, TWT: left ventricular total wall thickness.

Diastolic function in relation to rejection and IL-2 mRNA expression

A large individual variation was observed in the echocardiographic measurements. No differences in the echocardiographic variables were observed at the group level between AR+ and AR- EMB (Table 3a), between biopsies with or without IL-2 mRNA expression (Table 3a), or between the subdivided AR positive and AR negative samples in biopsies with or without IL-2 mRNA expression (Table 3b).

Table 3b: *Echocardiographic parameters at the time of EMB with (AR+) and without (AR-) acute rejection subdivided in samples with (IL-2+) and without (IL-2-) IL-2 mRNA expression.*

	AR- / IL-2-	*AR- / IL-2+*	*p-value*	*AR+ / IL-2-*	*AR+ / IL-2+*	*p-value*
TWT cm, mean (SD)	2.16 (0.30)	2.05 (0.30)	0.13	2.02 (0.14)	2.19 (0.31)	0.12
E m/sec, mean (SD)	0.82 (0.19)	0.84 (0.28)	0.66	0.71 (0.28)	0.87 (0.17)	0.13
E/A ratio, mean (SD)	2.22 (0.73)	2.14 (0.63)	0.68	1.70 (0.54)	2.18 (0.86)	0.25
DET msec, mean (SD)	161.5 (31.3)	175.2 (30.4)	0.11	143.3 (6.5)	156.1 (37.3)	0.32
IRP msec, mean (SD)	64.69 (19.50)	65.22 (21.60)	0.92	64.64 (26.16)	74.17 (12.07)	0.37

A: peak atrial mitral flow velocity, AR: acute rejection, DET: deceleration time of E, E: peak early mitral flow velocity, EMB: endomyocardial biopsy, IL-2: interleukin-2, IRP: isovolumetric relaxation period, TWT: left ventricular total wall thickness.

In the search for changes over time, differences in the echocardiographic parameters were calculated between the first postoperative measurement and the time of the first acute rejection or IL-2 mRNA expression. In this way, each patient served as his or her own control. Because the first echocardiographic measurement was used as the baseline, patients with acute rejection or IL-2 mRNA expression at the time of their first measurement (n = 2 and n = 4, respectively) were excluded from this specific analysis. Neither histological signs of acute rejection nor IL-2 mRNA expression alone was associated with individual changes over time in any of the studied echocardiographic parameters. However, by relating intragraft IL-2 positivity to echocardiographic changes over time after stratifying the data into AR+ and AR- time points, we found that IL-2 mRNA expression during the first acute rejection was significantly associated with increased left ventricular total wall thickness between the first postoperative measurement and the first acute rejection episode (Figure 1). In five of six patients with IL-2 mRNA expression during their first acute rejection, increased total wall thickness had preceded that rejection episode (mean (SD) change between the first postoperative measurement and the measurement during the first acute rejection episode was +0.22 (0.12) cm). In all patients without IL-2 mRNA expression during their first acute rejection, total wall thickness decreased (mean (SD) -0.18 (0.12) cm; p = 0.048, Fisher's exact test, Figure 1). In all non-rejecting control patients, total wall thickness

decreased during their first IL-2 positive EMB with respect to the first postoperative measurement (mean (SD) -0.30 (0.31) cm). The only patient in whom the total wall thickness had decreased preceding an IL-2 positive acute rejection episode had an exceptionally high total wall thickness of 2.68 cm during the first postoperative measurement, which might be attributable to the known history of hypertension of the donor. During the IL-2 positive rejection episode, the total wall thickness of this patient decreased to 2.32 cm, which is still relatively high.

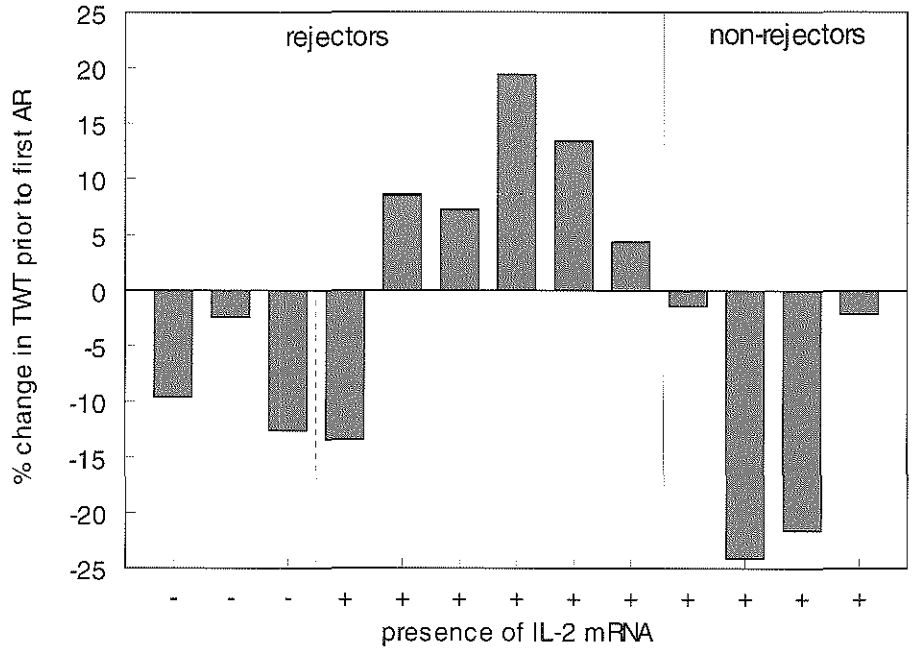

Figure 1: *Change in left ventricular total wall thickness (TWT) (%) from the first postoperative measurement to the first acute rejection (AR), in patients without (n = 3) and with (n = 6) Interleukin-2 (IL-2) mRNA expression during that first acute rejection (left), and towards the first IL-2 positive biopsy in non-rejecting controls (n=4; right), p=0.048, Fisher's exact test.*

Discussion

Diastolic function in relation to rejection

Numerous investigators have explored the clinical use of echocardiography to replace invasive EMB sampling for the diagnosis of acute rejection.[2,5,6,15,16] During acute rejection, high left atrial pressures combined with increased stiffness of the myocardium

can lead to a number of echocardiographic changes such as early opening of the mitral valve (short isovolumetric relaxation period); rapid filling of the left ventricle during the passive filling phase in association with rapid increase of left ventricular pressure (high E and decreased deceleration time); and subsequent small contribution of atrial contraction to left ventricular filling (low A and increased E/A ratio).[4] Although some authors described an association between acute rejection and echocardiographic variables at the group level, the considerable overlap between individuals still limits replacement of EMB as the benchmark for diagnosis of acute rejection after heart transplantation.

In the present study, no direct association was found at the group level between the studied echocardiographic parameters and histological evidence of acute rejection alone. This is in agreement with earlier findings and can be explained by the influence of numerous factors. Besides the risk of false negative biopsies and the effect of the immune response, left ventricular filling properties and heart morphology can be affected by loading conditions, hypertension, edema resulting from ischemia/reperfusion injury, and treatment with diuretic or vasodilators.[15-19] Moreover, it has been described that treatment with cyclosporin, in comparison with previous treatment strategies without calcineurin blockers, results in less pronounced morphologic and functional changes during acute rejection with loss of sensitivity of echocardiographic parameters.[20] Furthermore, diastolic function parameters improve in the early postoperative period, reflecting recovery from the operative insult, which may conceal acute changes caused by the rejection process.[4] Increased left ventricular mass in the absence of histological signs of acute rejection may also be the result of vascular (humoral) rejection, in which the cells of the microvasculature are target for injury early in the alloimmune response.[7] However, because vascular rejection was not determined routinely in EMB, we were unable to evaluate the contribution of vascular rejection (alone or in combination with cellular rejection) to left ventricular wall thickening in our patients.

IL-2 mRNA expression in relation to rejection

IL-2 is a key regulator of cellular immunity by its autocrine stimulation of T-cell maturation, differentiation, proliferation, and apoptosis.[10,21] In agreement with our

previous results, we found a strong relationship between local IL-2 mRNA expression in EMB and histological signs of acute rejection.[11] However, IL-2 mRNA is not always present during acute rejection and, inversely, acute rejection is not always diagnosed during the presence of IL-2 mRNA.[14,22] Several factors could explain this discrepancy. Firstly, as a result of sampling errors the diagnosis of acute rejection may be false negative. Secondly, because of the redundancy of the cytokine network, lymphocyte proliferation can be stimulated by alternative T cell growth-factors (e.g. IL-15) during inhibition of IL-2 mRNA expression by immunosuppressive therapy.[23] Thirdly, IL-2 mRNA expression may precede histological and clinical signs of acute rejection or IL-2 mRNA can be expressed by a low number of infiltrated lymphocytes in absence of myocyte damage (low grade rejection).[24] Besides, although intragraft cytokine measurements do not automatically reflect peripheral events, we cannot entirely exclude the influence of bacterial or viral infection episodes on IL-2 mRNA expression within the graft. Finally, high concentrations of local IL-2 may trigger apoptosis of activated lymphocytes, thereby downregulating the immune response.[21]

Diastolic function in relation to IL-2 mRNA expression

New onset left ventricular dysfunction after heart transplantation may be caused by the presence and cytotoxic effects of infiltrated immune cells, but may also depend on the production of cytokines (e.g. IL-2) by these infiltrating cells.[25] Cardiac complications are a common side effect of recombinant IL-2 immunotherapy in humans.[13] Moreover, in the transplant setting, high IL-2 serum levels have been associated with impaired hemodynamics but were not correlated with impaired diastolic graft function as detected by echocardiography.[26] Valantine and colleagues[27] found no relation between IL-2 mRNA expression in EMB and acute rejection or allograft dysfunction. However, the measured IL-2 mRNA expression levels in their study were extremely low, probably because of the in situ hybridization technique that they used.[27]

In the present study, we found a significant relation between IL-2 positive acute rejection and increased left ventricular total wall thickness. This increase was not seen in patients with IL-2 positive AR- EMB, or in patients with IL-2 negative acute rejections. We speculate that the IL-2 expression during acute rejection in our patient group may reflect the severity of the inflammation process during acute rejection. We did not

observe a similar association with the measured functional parameters (E, A, deceleration time and isovolumetric relaxation period), probably because of the large influence of loading conditions and blood pressure on these parameters. Our previous finding that IL-2 mRNA expression during the first acute rejection is associated with early development of graft vascular disease[14] supports the hypothesis that IL-2 positive acute rejection episodes are more serious than IL-2 negative acute rejections. Because vascular leakage is a known side effect of IL-2 immunotherapy, it is likely that the wall thickening as seen during the IL-2 positive rejection episodes is the result of edema.[28] IL-2 may lead to edema by stimulating the production of inducible nitric oxide synthase (iNOS), leading to overproduction of nitric oxide, which has been reported to cause capillary damage followed by fluid leakage.[29]

In summary, our results show a significant increase of left ventricular total wall thickness before IL-2 positive acute rejection. Whether vascular rejection plays a part in this process remains to be determined. We conclude that rejection accompanied by intragraft IL-2 mRNA expression has a greater influence on heart morphology and therefore may be indicative for more severe rejection.

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CHAPTER 7

APOPTOTIC DEATH OF INFILTRATING CELLS IN HUMAN CARDIAC ALLOGRAFTS IS REGULATED BY IL-2, FASL AND FLIP

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Submitted.

Abstract

After transplantation unrestricted proliferation of alloreactive T cells might be prevented by activation induced cell death (AICD). *In vitro* studies have shown that AICD is triggered by a IL-2-dependent activation of the Fas-FasL pathway and that this pathway can be inhibited by FLICE inhibitory protein (FLIP). To define whether IL-2, FasL and FLIP regulate the apoptotic cell death of graft infiltrating T cells during cardiac rejection, we stained frozen tissue slices of endomyocardial biopsies from cardiac allograft recipients for CD3 (T cells), for DNA strand breaks (TUNEL assay), and for FLIP protein to characterize the nature and number of apoptotic cells. Subsequently, we measured intragraft IL-2, FasL, and FLIP mRNA expression levels in endomyocardial biopsies sampled before, during, and after acute cellular rejection by real-time RT-PCR. In our endomyocardial biopsies apoptosis mainly occurred in CD3 positive T cell infiltrates. The number of TUNEL stained mononuclear cells was inversely correlated with FLIP mRNA expression levels ($p = 0.09$). FLIP-protein was present in 5-10% of the infiltrating cells and was constitutively produced by cardiomyocytes irrespective of the rejection grade. Rejection biopsies had elevated IL-2 and FasL mRNA expression levels compared to the expression levels before and after acute rejection ($p = 0.03$ and $p = 0.11$ respectively), while FLIP mRNA expression levels were significantly decreased during rejection ($p = 0.05$). In conclusion, our results indicate that during the IL-2 induced rejection process, infiltrated T cells become more sensitive to apoptosis in an attempt to reduce the immune response against the allograft.

Introduction

Apoptotic death of activated lymphocytes is an important mechanism to maintain T cell homeostasis. This has clearly been demonstrated in apoptosis-deficient mice that show lymphadenopathy and autoimmunity associated with increased survival of autoreactive CD4+ T cells and a defect in peripheral T-cell deletion.[1] There are two distinct mechanisms of mature T cell apoptosis.[2] The first is called passive cell death which occurs when activated T cells are deprived of growth factors (cytokine withdrawal). This mechanism is responsible for removal of expanded T cells after antigen clearance. The second pathway of T cell apoptosis is activation induced cell death (AICD) which is induced by repeated antigen stimulation. AICD prevents unrestricted proliferation of

activated T cells in the presence of antigen. In the transplant setting, AICD of alloreactive T cells may account for a downregulation of the immune response against the transplanted organ. Therefore, a better understanding of this process may provide the basis for tolerance induction after organ transplantation.[3,4,5]

AICD is mediated by the interaction of cell surface Fas (CD95) with its corresponding ligand (FasL). This Fas-mediated AICD is the principal apoptotic pathway of CD4+ cells, while other death-receptors (TNF-R, TRAIL-R, DR3 and DR6) may contribute to AICD of CD8+ T cells.[1] Fas is constitutively expressed on T cells but the expression of FasL can be rapidly induced upon activation.[6] Ligation of cell surface Fas by FasL on the same cell or on neighbour cells results in oligomerization of Fas and leads to the recruitment of the adaptor protein FADD (Fas associated death domain protein) to the cytoplasmic death domain of the receptor. Caspase 8 (also called Fas-like Interleukin-1β-converting enzyme, FLICE) binds to FADD, and is subsequently activated by self-cleavage. Caspase 8 promotes direct cleavage of various downstream caspases, such as caspase 3, caspase 6, and caspase 7, which in turn leads to apoptotic cell death.[7] This Fas-mediated cell death is tightly controled by apoptosis inhibitors interfering at various levels with the apoptosis cascade.[2] The best defined upstream inhibitor of AICD is FLICE-inhibitory protein (FLIP).[8] This protein was first identified as viral protein (v-FLIP) but soon the mammalian equivalent (c-FLIP) had been recognized.[9] Several splicing isoforms of FLIP exist, two of which are expressed as proteins in vivo: short FLIP ($FLIP_S$) and long FLIP ($FLIP_L$).[9] FLIP is structurally similar to caspase 8 but lacks the cysteine residue important for catalytic activity. Owing to this structural homology, FLIP interferes with the activation of caspase 8 by competing for binding to the death receptor complex, although recently it has been suggested that low concentrations of FLIPL can also promote apoptosis.[10]

Interleukin-2 (IL-2) is thought to play a dualistic role during the life-cycle of T cells. This was revealed by the finding that knockout mice lacking IL-2 or the IL-2-receptor develop lymphoproliferative diseases rather than immunodeficiency, which contradicts the traditional role of IL-2 as T cell growth factor.[1,11] It is thought that early in an immune response, IL-2 stimulates the proliferation and differentiation of T cells and is required for their clonal expansion. However, as the response develops and the amount of IL-2 increases, activated T cells are primed for AICD, which serves as a critical feedback

mechanism to maintain T cell homeostasis.[2,7,12] Although the growth-promoting function of IL-2 can be replaced by other cytokines of the IL-2-receptor family, the AICD-potentiating effect of IL-2 can not be compensated by these cytokines.[12]

After transplantation IL-2 in its traditional role stimulates proliferation and differentiation of alloreactive T cells. The presence of IL-2 in allografts has often been associated with the occurence of acute cellular rejection, and recently we showed that high IL-2 mRNA expression during acute rejection was associated with increased left ventricular total wall thickness.[13,14] On the other hand, IL-2 might also be important for priming alloreactive T cells for apoptosis and thereby enhancing tolerance against the allograft.[12] The allograft is a potential site for AICD because considerable quantities of antigen and large numbers of IL-2 producing T cells are present.[15-17] We hypothesize that during rejection of transplanted human hearts apoptosis of infiltrated alloreactive T cells occurs, which is regulated by IL-2, FasL and FLIP. Therefore, we characterized infiltrating cells in endomyocardial biopsies for the number of apoptotic cells and for the presence of FLIP protein and subsequently measured intragraft IL-2, FasL, and FLIP mRNA expression levels before, during, and after acute cardiac allograft rejection.

Material and methods

Patients and material

We studied 26 cardiac allograft reciepients. Maintenance immunosuppressive therapy consisted of cyclosporine A and low dose steroids alone (n=15), or with additional MMF (triple therapy, n=11). For immunohistochemistry we studied 49 endomyocardial biopsies sampled during the first three months post-transplant from 15 recipients. Additionally, we studied 38 endomyocardial biopsies for mRNA expression levels of IL-2, FasL, and FLIP. These biopsies were sampled before acute rejection, during acute rejection, and after successful anti-rejection therapy with bolus i.v. steroids. Acute rejection was diagnosed by histological assessment of myocardial biopsies and graded according to the guidelines of the International Society for Heart and Lung Transplantation (ISHLT).[17] Patients with ISHLT rejection grade >2 were considered to have an acute rejection and received additional immunosuppressive treatment.

Immunohistochemistry of CD3 and FLIP

Frozen tissue specimens were cut in 5 μm sections, air dried, and fixed in aceton. For T cell staining, the sections were incubated for 30 min with CD3 monoclonal antibody (1:10) (Beckton Dickinson, Lexington, KT, USA). For detection of CD3 positive cells we used the alkaline-phosphatase-anti-alkaline-phosphatase (APAAP) method. Briefly, the sections were rinsed in phosphate-buffered saline (PBS) and incubated for 30 min with rabbit anti-mouse IgG (1:20) (Dako, Glostup, Denmark) followed by 30 min incubation with APAAP-mouse complex (1:50) (Serotec, Oxford, UK) in PBS/5%BSA. Red precipitation was obtained by incubation in the dark with new fuchsine as described by the manufacturer. In control sections the primary antibody was omitted. Sections were counterstained with Mayer's hematoxylin. The number of CD3 positive cells was expressed per half field at 100x magnification (objective 10x). The biopsy sections were scored on a scale from 0 to 4: score 0: no CD3 positive cells, score 1: 1-10 CD3 positive cells, score 2: 11-30 CD3 positive cells, score 3: 31-100 CD3 positive cells, score 4: >100 CD3 positive cells.[18]

For FLIP staining, the sections were incubated overnight at 4°C with primary antibody against FLIP (1:5) (Sanvertech, Santa Cruz, CA, USA), detecting both $FLIP_S$ and $FLIP_L$. Control sections were incubated with isotype mouse IgG1 antbody (1:2,5) (Dako). FLIP was visualized by 30 min. incubation with Powervision mouse (Klinipath, Duiven, The Netherlands), followed by 10 min incubation with DAB. Sections were counterstained with Mayer's hematoxylin.

TUNEL staining

For TUNEL staining, frozen tissue specimens were cut in 5 μm sections and air dried. The sections were fixed in 4% phosphate buffered formalin, washed in PBS, and incubated with 0.3% H_2O_2 in PBS for 10 min to block endogeneous peroxidases. Subsequently the sections were preincubated for 5 min in a buffer containing 0.5 M sodiumcacodylaat (Merck, Darmstadt, Germany), 1 mM cobaltchloride (Merck), 1 M dithiotreitol (Sigma, St. Louis, MI, USA), 0.05% bovine serum albumine (Sigma), and 0.15 M sodiumchloride (Sigma). To stain DNA strand-breaks, the sections were incubated for 2.5 hour at 37°C with biotinylated dUTP (Roche, Mannheim, Germany) in the presence of 0.25 e.u./μl terminal deoxydinucleotidyl transferase (TdT; Promega,

Madison, WI, USA). The reaction was terminated by transferring the slides to PBS/0.5% Tween for 15 min at room temperature. For detection of TUNEL positive cell nuclei, sections were incubated with horseradish peroxidase labelled streptavidine (1:50) (Dako) and subsequently incubated for 7 min with diaminobenzidine (Fluka, Buchs, Switzerland) resulting in brown stained nuclei. Sections were counterstained with Mayer's hematoxyline. The number of TUNEL positive cell nuclei was expressed per field at 400x magnification (objective 40x). The biopsy sections were scored on a scale from 0-3: score 0: no TUNEL positive cells; score 1: 1-5 TUNEL positive cells, score 2: 6-10 TUNEL positive cells, score 3: >10 TUNEL positive cells.

mRNA detection by real-time RT-PCR

We used real-time reverse transcriptase polymerase chain reaction (real-time RT-PCR) for quantitative measurement of IL-2, FasL, FLIP, and the constitutively expressed housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA expression in endomyocardial biopsies. We used primer express software (Applied Biosystems, Norwalk, CT, USA) to select sequence-specific primers and probes for IL-2 and FLIP (detecting both $FLIP_S$ and $FLIP_L$). IL-2 sense primer: 5'-TTT-GAA-TGG-AAT-TAA-TTA-CAA-GAA-TCC-3', IL-2 antisense primer: 5'-TTC-TAG-ACA-CTG-AAG-CTG-TTT-CAG-TTC-3', IL-2 probe: 5'-CCA-GGA-TGC-TCA-CAT-TTA-AGT-TTT-ACA-TGC-CC-3'. FLIP sense primer: 5'-AGG-CAA-GAT-AAG-CAA-GGA-GAA-GAG-T-3'; FLIP anti-sense primer: 5'-TTT-TCT-AAT-AAA-TCC-AGT-TGA-TCT-GGG-3', FLIP probe: 5'-TCT-TGG-ACC-TTG-TGG-TTG-AGT-TGG-AGA-AA-3'. Pre-developed TaqMan® assays (PDAR) were used to measure FasL and GAPDH concentrations (Applied Biosystems).

Total RNA was extracted from snapfrozen endomyocardial biopsies, and cDNA was synthesized with random primers as described previously in detail.[19] Positive control samples were produced by messenger RNA extraction and cDNA synthesis from 10^6 human spleen cells stimulated with 1% phytohemagglutin-M (PHA; Difco, Detroit MI, USA) for 24 hours at 37°C. Negative control samples consisted of diethyl pyrocarbonate-treated H_2O as no-template reaction. All experiments were performed in duplicate.

Five μl cDNA was added to 45 μl PCR mixture containing 25 μl Universal PCR Master Mix (Applied Biosystems), 1 μl sense primer (50 pmol), 1 μl antisense primer (50 pmol),

1 μl probe (10 pmol), and 17 μl DEPC-treated H_2O. PCR was performed using the ABI Prism 7700 sequence detector (Applied Biosystems). PCR conditions were 2 min 50°C, 10-minute 94°C denaturation, followed by 40 cycles of 15 sec denaturation at 95°C and 1 min annealing at optimal temperatures for IL-2 (58°C), FasL (60°C), FLIP (59°C), or GAPDH (60°C). A standard curve with serial dilutions of known amount of standard RNA was used to determine the initial target mRNA concentrations in biopsies. The relative concentration of intragraft IL-2, FasL and FLIP mRNA was divided by the relative concentration of GAPDH mRNA to indicate the mRNA expression level normalized for the amount of mRNA used for RT-reaction and for the efficacy of each reaction. Biopsy specimens negative for the house-keeping gene GAPDH mRNA expression were excluded from further analysis.

Statistics

Differences in median mRNA expression levels between EMB sampled before, during, and after acute rejection were analyzed by nonparametric Friedman repeated measures analysis of variance (ANOVA). Correlation between FLIP mRNA expression levels and TUNEL score was analyzed by nonparametric Kruskal-Wallis ANOVA. Associations with $p < 0.05$ were considered statistically significant.

Table 1: *ISHLT rejection grades, CD3 and TUNEL scores (as explained in material and methods section) in 49 endomyocardial biopsies sampled from 15 consecutive heart transplant patients.*

patient number	weeks post-transplant	rejection score (ISHLT)[16]	CD3 score (see text)	TUNEL score (see text)	TUNEL positive myocytes
1	8	0	2	2	yes
1	12	0	1	1	no
2	1	0	1	1	no
2	3	1A	3	3	no
2	4	0	1	1	no
2	8	1A	-	2	no
2	10	3A	3	3	no
3	1	0	2	3	no
3	4	1A	3	3	no
3	5	0	3	2	no
3	10	3A	2	2	no
3	11	3A	3	3	no
3	13	1A	2	1	no
4	5	3A	2	1	no
4	6	1A	2	3	no
5	2	0	1	1	no
5	4	1A	2	2	yes
5	5	3A	4	2	no
5	6	0	3	3	no
6	2	0	1	2	yes
6	3	1A	0	2	yes
6	4	0	4	1	no
6	5	3A	3	0	no
7	3	3A	2	3	no
7	4	1A	1	2	no
7	10	1A	2	1	no
7	14	3A	2	1	yes
8	2	1A	2	1	no
8	3	3A	3	2	no
8	4	3A	3	1	no
8	9	1A	2	1	no
8	14	3A	3	1	no
9	6	3A	2	1	no
10	4	3A	2	1	no
11	1	1A	2	1	no
11	2	1A	2	1	no
11	3	1A	3	1	no
11	5	3A	3	1	no
11	8	1A	2	1	no
11	10	1A	2	1	no
12	3	1A	1	1	no
13	6	0	1	1	no
13	7	0	1	1	no
14	4	0	1	1	yes
14	5	0	1	1	yes
14	8	0	2	2	no
14	10	0	1	2	no
15	9	0	2	0	no
15	15	0	1	0	no

CD3 score = number of potitive cells in half 100 x field: 0 = none; 1 = 1-10; 2 = 11-30; 3 = 31-100; 4 >100; Tunel score = number of positive cells in 400 x field : 0, none; 1 = 1-5; 2 = 6-10; 3 >10.

Results

Presence of apoptotic cells in endomyocardial biopsies

We studied 49 endomyocardial biopsies (ISHLT rejection grade 0 n = 18, ISHLT rejection grade 1A n = 17, ISHLT rejection grade 3A n = 14) to determine the presence and number of apoptotic cells (Table 1). In only 7/49 biopsies we detected TUNEL positive cardiac myocytes (range 1-7 cells/400x field) but their number was not associated with acute rejection. In contrast, we found strong TUNEL staining in mononuclear cell infiltrates. Figure 1 shows an example of two sequential biopsies from one patient. The left panels show a biopsy sampled in the second week posttransplant with ISHLT rejection grade 1A in which the CD3 positive infiltrate shows a small proportion of TUNEL positive cells (panels A and C). The right panels show the biopsy sampled in the subsequent week with ISHLT rejection grade 3A in which a significant number of TUNEL positive infiltrated cells are present (panels B and D). Median number of TUNEL positive cells was 1-5 cells per 400x field (TUNEL score 1, Table 1), which corresponded approximately with 15% of the infiltrated mononuclear cells (data not shown). The number of apoptotic infiltrating cells did not correlate to the ISHLT rejection score (Table 1).

Mechanism of apoptosis during acute rejection

To determine whether FLIP protein could be produced by infiltrated cells we stained endomyocardial biopsies for FLIP protein expression. FLIP protein was present in 5-10% of infiltrated mononuclear cells and was constitutively produced at strong intensity by cardiomyocytes (Figure 1, panels E-H). We did not observe quantifiable differences in the intensity of FLIP protein expression in mononuclear cells or cardiomyocytes between the individual biopsies. At the mRNA level FLIP expression in these biopsies revealed an inverse correlation with the number of apoptotic infiltrated cells ($p = 0.09$). A higher number of apoptotic infiltrated cells was associated with lower mRNA expression levels of the apoptosis inhibitor FLIP (Figure 2).

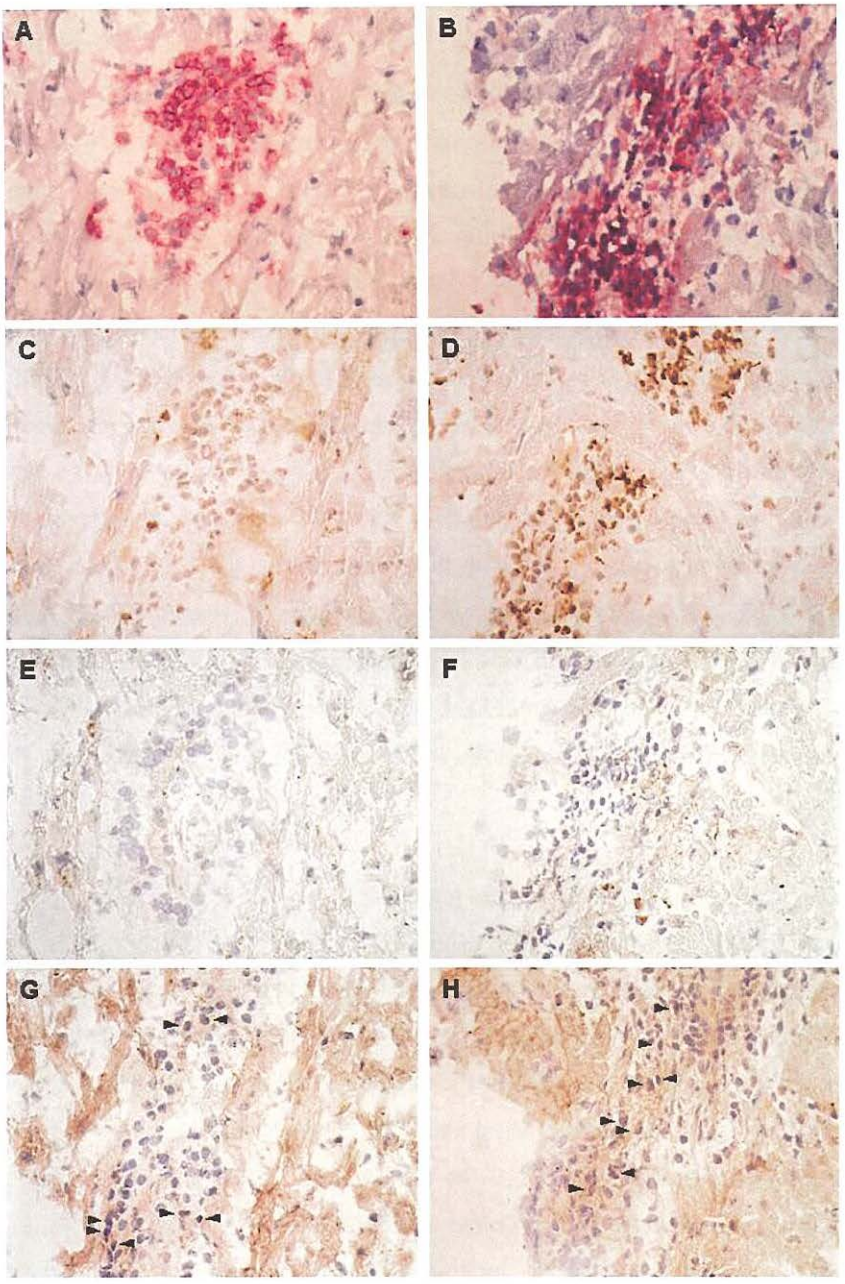

Figure 1: *Example of CD3 (panel A and B), TUNEL staining (panel C and D), FLIP isotype control (panel E and F), and FLIP protein (panel G and H) of an endomyocardial biopsy sampled from the same patient before acute rejection (left panels A, C, E, and G: ISHLT rejection grade 1A) and during acute rejection one week later (right panels B, D, F, and H: ISHLT rejection grade 3A). Infiltrating cells positive for FLIP protein are indicated by arrowheads in panel G and H.*

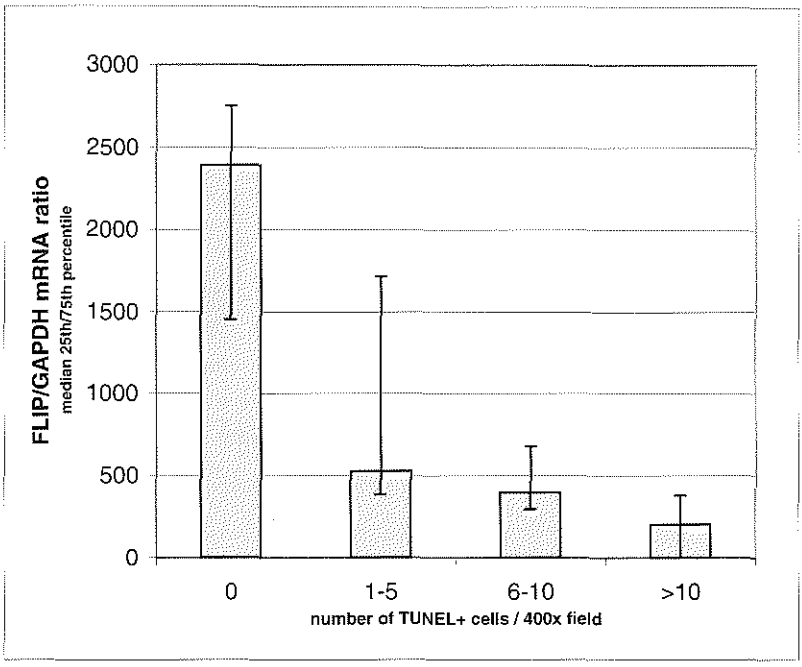

Figure 2: *Median FLIP/GAPDH mRNA expression levels in biopsies with increasing TUNEL staining score.*

To assess the mechanism of apoptosis regulation during acute rejection, we measured IL-2, FasL, and FLIP mRNA expression in 39 endomyocardial biopsies sampled before acute rejection, during acute rejection, and after successful rejection therapy. Figure 3a shows that intragraft IL-2 mRNA expression levels were increased during acute rejection. Median relative IL-2 mRNA expression before, during, and after rejection was respectively 0.1, 0.4, and 0.1 ($p = 0.03$). Figure 3b shows that the expression level of FasL mRNA expression was 10-fold higher than the expression level of IL-2 and was increased during and after rejection compared to the level before rejection. Median relative FasL mRNA expression before, during, and after rejection was respectively 5, 13, and 12 ($p = 0.11$). In contrast, FLIP mRNA expression levels were 1000-fold higher than IL-2 mRNA expression levels, and decreased during acute rejection compared with the expression levels before and after acute rejection (Figure 3c). Median relative FLIP mRNA expression before during and after AR was respectively 3580, 2430, and 3170 ($p = 0.05$).

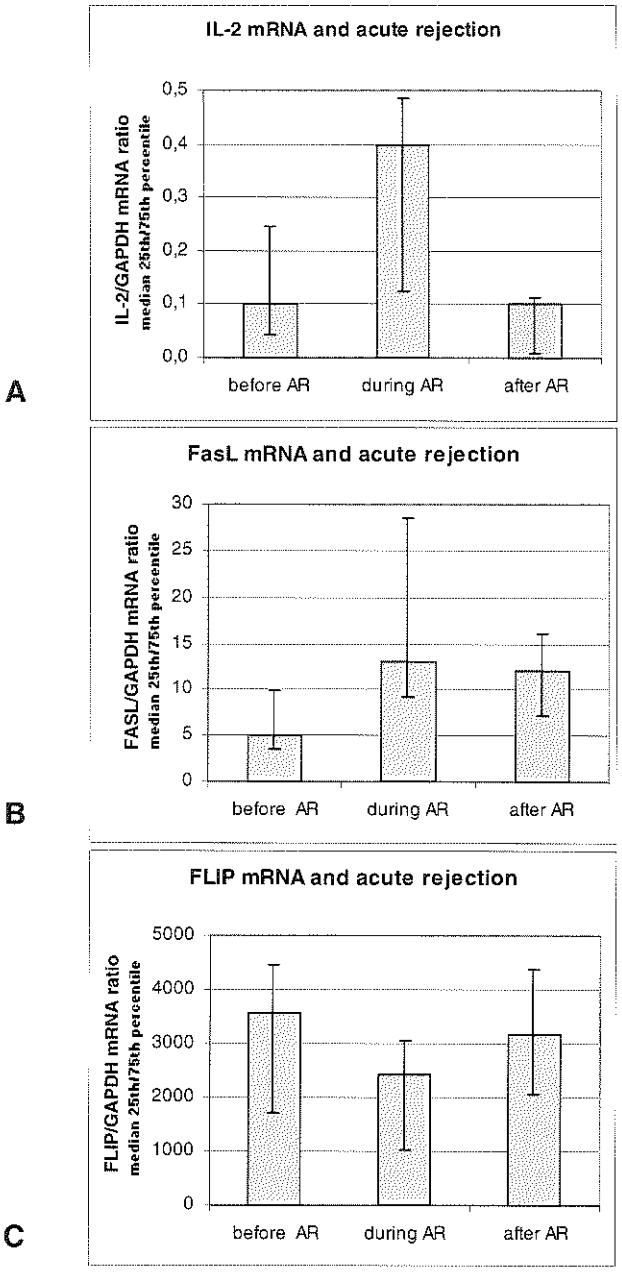

Figure 3: *Median IL-2 (A; p = 0.03), FasL (B; p = 0.11), and FLIP (C; p = 0.05) mRNA expression level in endomyocardial biopsies before, during and after histological signs of acute rejection.*

Discussion

In this study we analyzed endomyocardial biopsies for the degree of T cell apoptosis as well as for the intragraft expression levels of potential apoptosis-regulators: IL-2, FasL, and FLIP. Apoptosis after heart transplantation may play a role both in tissue damage during allograft rejection (apoptosis of cardiac myocytes and endothelial cells) and in downregulation of the immune response (apoptosis of T cells).[4] Conflicting data have been published on the rate of cardiac myocyte apoptosis in allograft rejection.[20] While some studies demonstrated a correlation between apoptosis of cardiac myocytes and acute rejection.[21,22] others did show that cardiac myocyte apoptosis was not associated with the degree of rejection.[23,24] Apoptosis of infiltrated cells, on the other hand, has frequently been observed in experimental transplant models.[23-25] Moreover, in a study in human transplant recipients it has been shown that 40-60% of infiltrated cells in endomyocardial biopsies was apoptotic. Double staining revealed that these were mainly CD4+ T cells, and that their number correlated with the histological rejection grade.[16] In our endomyocardial biopsies, we found very few apoptotic cardiac myocytes, whereas approximately 15% of the mononuclear cell infitrate was apoptotic. However, the number of TUNEL positive infiltrated cells was not related to the ISHLT rejection score. This is most likely because apoptotic infiltrated cells were not only present during acute rejection, but also after successful acute rejection therapy.

Immune tolerance can be induced by a number of T cell down-regulating mechanisms, including clonal deletion, anergy, suppression, and AICD.[26] A number of studies have shown that AICD is involved in spontaneous allograft acceptance and that IL-2 is necessary for this process.[4,5,25] In IL-2 knockout mice, for example, tolerance induction could not been achieved.[3] Furthermore, in a rat transplant model, kidney and liver allograft acceptance was associated with increased expression of IL-2 and exhaustive activation and differentiation of alloreactive T cells.[27] However, a problem with tolerance induction in the clinical situation could be the fact that conventional immunosuppression interferes with the IL-2 pathway and thereby inhibits AICD.[4,24] For instance, in a recent study we observed that treatment with anti-CD25 mAb downregulates both IL-2 and FasL and inhibits apoptosis of graft infiltrating T cells.[28]

There are two mechanisms by which IL-2 may induce AICD. First, IL-2-mediated activation of STAT5 or NF-κB enhances the transcription and expression of FasL in

activated T cells. Second, IL-2 inhibits the transcription of the apoptosis inhibitor FLIP.[7,12] The molecular regulation of FLIP expression still remains unknown although some data suggest that FasL and FLIP expression may use common regulatory pathways.[29] Support for a regulatory model in which IL-2 upregulates FasL and downregulates FLIP has been provided by studies in IL-2 knockout mice. These animals fail to downregulate FLIP upon T cell stimulation, which could be restored by additing exogenous IL-2.[7,9] Moreover, in vitro experiments show that resting T cells as well as proliferating T cells early after activation are resistant to apoptosis to allow the immune reaction to occur, while most of the activated T cells become increasingly sensitive to AICD when they are re-stimulated via the T-cell receptor (TCR).[2,9,30] This change in sensitivity to AICD is accompanied by decreased FLIP expression levels.[9] In mixed lymphocytes reactions (MLR), early expression of cell-surface FasL has been detected along with high expression of the apoptosis inhibitor FLIP. The concentration of IL-2 increases to peak at 3 days following primary antigen-presentation. After 5 days, FLIP expression is downregulated and these cells become sensitive to AICD. FLIP levels are markedly reduced after 7 days in culture, which coincides with maximal sensitivity to Fas-mediated apoptosis.[12,30] The clinical relevance of FLIP in regulating immune responses has recently been demonstrated in patients with the autoimmune disease MS, in whom overexpression of FLIP was associated with severity of the disease.[31]

In the present study, we found increased IL-2 mRNA expression levels in endomyocardial biopsies sampled during rejection. This was accompanied by increased FasL and decreased FLIP mRNA expression levels. The increased IL-2 mRNA expression during acute rejection reflects T cell activation that, according the mechanism described above, will result in increased FasL and decreased FLIP mRNA expression levels in the T cells. Immunohistochemically we showed that the cardiac myocytes constitively contain abundant FLIP protein. This suggest that cardiomyocytes constitively express FLIP mRNA that accounts for the high FLIP mRNA expression levels in the biopsies (10 times higher compared to FAS-L and IL-2). This high level of cardiomyocyte FLIP mRNA makes the interpretation of the FLIP mRNA data difficult. However, the level of FLIP protein was not affected by the rejection status of the biopsies and we did not find significant changes in the number of apoptotic myocytes before and during rejection. It is therefore unlikely that the downregulation of FLIP mRNA is attributable to changes in

the expression level in the cardiomyocytes. The observation that 5-10% of the infiltrated cells are producing FLIP protein, and that a high number of TUNEL positive infiltrated cells was correlated with low FLIP mRNA levels, supports the hypothesis that apoptosis-sensitive infiltrated cells are responsible for the downregulation of FLIP mRNA expression levels as shown during acute rejection.

In our clinical tansplant setting, we showed that T-cell apoptosis during cardiac allograft rejection is accompanied by increased intragraft IL-2 and FasL mRNA expression levels and decreased FLIP mRNA expression levels. This suggests that during the IL-2 induced rejection process, infiltrated T cells become sensitive to apoptosis in an attempt to downregulate the immune response, which could lead to a more tolerant state toward the allograft.

Acknowledgements

We like to thank Mariska Klepper, Sandra van den Engel, and Dick van Wichen for their skillful technical assistance.

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CHAPTER 8

SUMMARY AND CONCLUSIONS

Summary and conclusions

Summary of the results

Allo-antigen (in)dependent events early after heart transplantation may determine later graft outcome. In this thesis, the role of intragraft cytokines in the sequence of events that lead to rejection and/or graft dysfunction after clinical heart transplantation is reported.

Chapter 1 is a general introduction into the allo-antigen dependent and independent inflammatory response that is initiated after clinical heart transplantation. A description of the different types of rejection, the role of cytokines, changes in cardiac function, and the role of T cell apoptosis in decreasing the immune response is given.

Chapter 2 reveals that intragraft cytokine mRNA expression levels of monocyte chemoattractant protein-1 (MCP-1) and basic fibroblast growth factor (bFGF) are higher in biopsies sampled during the transplantation procedure compared with biopsies sampled in the first week. The expression levels of these cytokines are not related to length of ischemic times, immunosuppressive induction treatment, cyclosporine trough levels, or the development of graft vascular disease in the first year. However, patients with a high incidence of acute rejection have higher bFGF mRNA expression levels in their first week biopsy.

The first part of **Chapter 3** demonstrates that intragraft expression of transforming growth factor-β (TGF-β) and platelet derived growth factor-A (PDGF-A) at 9-months posttransplant, but not at 1-month, is higher in patients who develop graft vascular disease within the first year. The data in the second part show that cyclosporine trough levels are not associated with TGF-β mRNA expression levels. On the contrary, higher cyclosporine trough levels are associated with lower PDGF-A mRNA expression at 9 months post-transplant. Nonetheless, cyclosporine trough levels at 9-month post-transplant did not differ between patients with and without graft vascular disease at one year.

Chapter 4 reveals that intragraft mRNA expression levels of cytokines involved in the response-to-injury (tumor necrosis factor-α (TNF-α), MCP-1, TGF-β, PDGF-A, and bFGF) are gradually down-regulated during the first three post-operative months. None of these cytokines are predictive for histological signs of acute cellular rejection in the next endomyocardial biopsy. However, the presence of acute rejection is strongly associated

with higher TNF-α mRNA expression levels. After acute rejection the majority of patients show increased mRNA expression levels of PDGF-A and bFGF.

Chapter 5 shows that echocardiographic measurements of left ventricular wall thickness and echo-doppler measurements of left ventricular diastolic function are not associated with histological signs of acute rejection. Furthermore, this chapter demonstrates that the decrease in cytokine mRNA expression levels during the first three months post-transplant (as described in Chapter 4) is accompanied by an improvement in echo-dopplercardiographic parameters. However, no straightforward relationship exists between a specific cytokine mRNA expression pattern and the echocardiographic measurements of graft dimensions and function.

Chapter 6 confirms that histological signs of acute rejection are associated with intragraft interleukin-2 (IL-2) mRNA expression. The sole presence of IL-2 mRNA in biopsies is not associated with left ventricular dimension or diastolic function. Nonetheless, our data reveal that an increase in left ventricular total wall thickness precedes IL-2 positive acute rejection.

Chapter 7 provides evidence for the presence of apoptotic mononuclear cells in cardiac biopsies and on the other hand shows that mononuclear cells are able to produce the apoptosis inhibitor FLIP (FLICE inhibitory protein). Furthermore, it reveals that the elevated IL-2 mRNA expression levels in the graft during rejection is accompanied by increased mRNA expression levels of the apoptosis inducing ligand FasL and decreased mRNA expression of the apoptosis inhibitor FLIP.

General conclusions

Thus, immediately after clinical heart transplantation a broad range of cytokines in the graft is upregulated presumably because allo-antigen independent events trigger the response-to-injury mechanism. Surprisingly, the level of these cytokines is not related to the duration of ischemia or to the type or level of immunosuppressive therapy, indicating that other early events (brain death, surgical damage, reperfusion) may be more important in determining the initial intragraft cytokine expression level. Besides, the degree of response-to-injury triggered by these events may be regulated by a patient-specific balance between injury and protective factors at the endothelial level.

The early increase of growth factor gene expression (TGF-β, PDGF-A, bFGF) is not associated with later development of graft vascular disease, but in the case of bFGF rather with a higher incidence of acute rejection. This indicates that patients who fail to down-regulate their bFGF mRNA expression early after transplantation are at higher risk for acute rejection. The inhibition of the allo-antigen independent inflammatory response, therefore, could be an effective way to decrease the subsequent allo-antigen dependent immune response resulting in acute allograft rejection. Later after transplantation, gene expression of TGF-β and PDGF-A is upregulated during the development of graft vascular disease. This implies that local production of PDGF-A and TGF-β contribute to the pathogenesis of graft vascular disease after heart transplantation. Cytokine expression levels gradually decline during the first months after transplantation, which is accompanied by improved graft function. But individual cytokine mRNA expression patterns are not directly related to deteriorated graft function as measured by echo-dopplercardiography. Thus, it seems that the improvement in diastolic left ventricular function is associated with a general reduction of inflammation within the allograft rather than with a specific cytokine expression pattern.
The presence of cytokine mRNA in endomyocardial biopsies is not predictive for histological signs of acute cellular rejection later on. Nevertheless, the upregulation of intragraft IL-2 and TNF-α during rejection indicate that these cytokines play important roles during acute cellular rejection. Acute rejection in the presence of high IL-2 mRNA expression is also indicative for hemodynamically more severe rejection as this is accompanied by increased left ventricular wall thickness. On the contrary, besides its role in stimulating the proliferation and differentiation of T cells, IL-2 is involved in downregulation of the immune response by triggering T cell apoptosis in the heart allograft.

Taken together, the studies described in this thesis emphasize the key role of intragraft cytokines in the regulation of acute and chronic rejection in heart transplant recipients. Furthermore, these studies illustrate that the immune response is regulated by a local network of cytokines and other immune mediators and that their interactions are important for the outcome after clinical heart transplantation. In the near future, the improvement of new molecular techniques such as multiplex real-time PCR and micro-

array will facilitate the measurement of multiple cytokines in small tissue samples. These techniques may help further elucidate the multifactorial nature of the allo-immune response in a way that patients with increased or decreased risk for rejection can be identified at an early stage.

CHAPTER 9

SAMENVATTING IN HET NEDERLANDS

Introductie

In 1967 werd de eerste harttransplantatie bij de mens uitgevoerd door Christiaan Barnard in Kaapstad, Zuid-Afrika. Inmiddels zijn er wereldwijd meer dan 57.000 harten getransplanteerd, waarvan ruim 400 in Rotterdam. De belangrijkste complicatie na harttransplantatie is afstoting hetgeen het resultaat is van de afweerreactie van de ontvanger tegen het niet-eigen donorhart. De belangrijkste vormen van afstoting zijn acute en chronische afstoting. Acute afstoting ontstaat binnen weken tot maanden na transplantatie en begint na herkenning van vreemde eiwitten van het donorhart (allo-antigenen) door witte bloedcellen (T cellen) van de ontvanger. Deze herkenning leidt tot de vorming van kleine boodschapper eiwitten (cytokines). Deze cytokines zetten T cellen aan tot vermeerdering en differentiatie waarbij gespecialiseerde T cellen (cytotoxische T cellen) worden gevormd die het hartweefsel beschadigen in een poging om het lichaamsvreemde weefsel op te ruimen. Acute afstoting na harttransplantatie leidt pas in een vergevorderd stadium tot klachten of klinische verschijnselen (zoals hartfalen, ritmestoornissen, of plotse dood). Daarom is het opsporen van acute afstoting in een vroeg stadium van levensbelang. De beste methode hiervoor is nog steeds histologisch onderzoek van hartbiopten die in het eerste jaar na transplantatie op gezette tijden worden afgenomen. Door in een vroeg stadium te behandelen (door middel van intensivering van de afweeronderdrukkende medicatie) wordt een verslechtering van de hartfunctie voorkomen.

Chronische afstoting na harttransplantatie komt tot uiting als versnelde coronairsclerose en wordt gekenmerkt door vernauwing van de kransslagader van het donorhart over de gehele lengte, met later ook vorming van 'gewone' atherosclerotische plaques. Dit proces ontwikkelt zich in de loop van maanden tot enkele jaren en uiteindelijk vertonen bijna alle getransplanteerde harten tekenen van versnelde coronairsclerose. Het wordt waarschijnlijk veroorzaakt door een complex samenspel van factoren resulterend in herhaaldelijke beschadigingen van de bloedvaten. Vroege allo-antigeen onafhankelijke factoren die tot schade leiden zijn bijvoorbeeld hersendood van de donor, ischemie in de tijd tussen de uitname van het hart bij de donor en inplantatie in de ontvanger, de operatie zelf, en het weer tot stand komen van de bloedstroom in de ontvanger na de operatie (reperfusie). Later spelen waarschijnlijk ook acute afstoting en infectie een rol. Al deze gebeurtenissen stimuleren de produktie van cytokines die het

ontstekingsproces in stand houden (response-to-injury) en de vermeerdering van onder andere gladde spiercellen aanwakkeren. In Rotterdam worden alle harttransplantatiepatiënten met behulp van coronair-arteriografie gescreend op de aanwezigheid van vernauwingen in de kransslagader na 1 en 4 jaar. Een definitieve behandeling van versnelde coronairsclerose is echter nog steeds niet mogelijk.

De pomp-functie van het getransplanteerde hart wordt regelmatig beoordeeld met behulp van echo-dopplercardiografie. Hierbij wordt met name gekeken naar de afmeting en wanddikte van de linker kamer en naar de stroomsnelheid van het bloed van de linker boezem naar de linker kamer tijdens de rustfase van het hart (diastolische functie). Aangezien acute rejectie gepaard gaat met infiltratie van witte bloedcellen, beschadiging van hartspiercellen en met vochtophoping in het hart, kan dit leiden tot veranderingen in de afmetingen en de diastolische functie van de linker kamer wat te zien is op een echocardiogram. Vandaar dat verschillende onderzoekers hebben bestudeerd of deze niet-belastende techniek te gebruiken is om acute afstoting op te sporen. Tot op heden is er echter nog geen non-invasieve bepaling gevonden die het nemen van hartbiopten zou kunnen vervangen. Er zijn aanwijzingen dat cytokines in staat zijn om de samentrekking van hartspiercellen te remmen. Daarom zou de lokale aanwezigheid van cytokines na transplantatie de functie van het donorhart kunnen beïnvloeden.

Een ander aspect van de immuunreactie dat onder invloed staat van cytokines is T cel apoptose. Apoptose is de term voor celdood waarbij de cel niet kapot gaat, maar in kleine deeltjes uiteenvalt en vervolgens netjes door andere cellen wordt opgeruimd. T cel apoptose is mogelijk betrokken bij het stimuleren van tolerantie van een ontvanger voor zijn transplantaat, omdat het de T cellen zou kunnen uitschakelen die specifiek gericht zijn tegen het donorweefsel.

Doel van het onderzoek

Het doel van de studies, beschreven in dit proefschrift, was om de rol van cytokines in de immuunreactie te bestuderen die na transplantatie tot afstoting en functieverlies van het getransplanteerde hart leidt. Voor deze studies zijn hartbiopten van transplantatiepatiënten onderzocht op de mate van gen-expressie van verschillende cytokines op uiteenlopende tijdstippen na transplantatie. Daarnaast is in een deel van

deze transplantatiepatiënten de wandikte en functie van de linkerkamer met behulp van echo-dopplercardiografie bepaald gedurende de eerste drie maanden na transplantatie. Deze gegevens zijn geanalyseerd in relatie tot ischemietijd, afweeronderdrukkende medicijnen, acute afstoting, en chronische afstoting.

Resultaten en conclusies

Direct na harttransplantatie wordt een grote verscheidenheid aan cytokines geproduceerd, waarschijnlijk gestimuleerd door verschillende allo-antigeen onafhankelijke factoren zoals hersendood van de donor, ischemie, de operatieve ingreep en reperfusie. Opvallend is dat de mate van cytokine expressie niet correleert met de ischemietijd, wat er op duidt dat de andere factoren mogelijk een belangrijkere rol spelen in de stimulatie van cytokines. Verder blijkt dat een hoge expressie van cytokines vroeg na transplantatie niet geassocieerd is met het ontstaan van vroege tekenen van versnelde coronairsclerose, maar in het geval van bFGF (basic fibroblast growth factor) wel met het krijgen van een hoger aantal acute afstotingen in het eerste jaar. Dit suggereert dat door remming van de allo-antigeen onafhankelijke ontstekingsreactie de daaropvolgende allo-antigeen afhankelijke immuunreactie kan worden verminderd. Op 9 maanden na transplantatie bestaat er wel een verband tussen de expressie van de cytokines TGF-β (transforming growth factor-β) en PDGF-A (platelet derived growth factor-A) in het transplantaat en het ontstaan van versnelde coronairsclerose op 1 jaar. Deze cytokines spelen dus waarschijnlijk een rol tijdens de ontwikkelingsfase van deze uiting van chronische rejectie.

Gedurende de eerste maanden na transplantatie neemt de gen-activatie van cytokines langzaam af. Hoewel dit samengaat met een verbetering van de hartfunctie bestaat er geen direct verband tussen de gemeten cytokines en transplantaatfunctie. Dit duidt er op dat een algehele vermindering van het onstekingsproces in het transplantaat tot zowel een verlaging van cytokine gen-expressie als tot een verbetering van de hartfunctie leidt. Noch cytokinepatronen, noch echocardiografische bepalingen kunnen histologische tekenen van afstoting voorspellen. Wel blijkt dat in biopten ten tijde van afstoting een hogere gen-expressie van IL-2 (interleukine-2) en TNF-α (tumor necrosis factor-α) voorkomt dan in biopten zonder afstoting. Bovendien gaat afstoting in aanwezigheid van veel IL-2 gepaard met een verdikking van de linker kamerwand. Dit

bevestigt de belangrijke regulerende functie van cytokines tijdens het afstotingsproces. Aan de andere kant blijkt IL-2 ook een rol te spelen bij het uitdoven van de immuunreactie door het stimuleren van apoptose van geactiveerde T cellen in het transplantaat.

In dit proefschrift komt de centrale rol van cytokines bij de regulatie van acute en chronische afstoting na harttransplantatie duidelijk naar voren. Verder blijkt dat de wisselwerking tussen de verschillende cytokines en andere immuun-mediatoren die in het getransplanteerde hart aanwezig zijn, belangrijk is voor het verloop van de immuunreactie die na transplantatie op gang komt. In de nabije toekomst zal de ontwikkeling van nieuwe moleculair biologische technieken het bepalen van meerdere cytokines tegelijk in kleine stukjes weefsel vergemakkelijken. Hiermee kan het evenwicht tussen de verschillende cytokines en andere factoren die de allo-immuunrespons beïnvloeden beter in kaart worden gebracht, zodat patiënten met een verhoogd danwel verlaagd risico op afstoting tijdig geïdentificeerd kunnen worden.

Curriculum Vitae

De auteur van dit proefschrift werd op 7 januari 1970 geboren in De Bilt. In 1988 behaalde zij het VWO diploma aan de Werkplaats-Kindergemeenschap in Bilthoven. Een jaar later begon zij met de studie Gezondheidswetenschappen aan de Rijksuniversiteit Limburg in Maastricht en koos na de propadeuse voor de afstudeerrichting Biologische Gezondheidkunde. Haar afstudeeronderzoek (het opzetten van een in vitro mutageniteitstest) heeft zij uitgevoerd bij de afdeling Moleculair Biologische Toxicologie van het Research Instituut Toxicologie (RITOX) in Utrecht onder begeleiding van Wendy Kappers en Sjeng Horbach. Na het behalen van haar doctoraal diploma in 1993 bleef zij bij dezelfde afdeling werkzaam als onderzoeker Pro Deo (het bepalen van gen-expressie van heat-shock eiwitten). Begin 1996 trad zij in dienst als wetenschappelijk medewerker bij TNO-Voeding in Zeist. Hier werkte zij achtereenvolgens bij de afdelingen Voedingsanalyse onder leiding van Hans Verhagen (literatuuronderzoek) en bij de afdeling Farmacologie onder leiding van Renger Witkamp (het opzetten van een weefselkweeklaboratorium voor in vitro hormoon-onderzoek). In 1997 trad zij als AIO in dienst bij de afdeling Inwendige Geneeskunde van het Academisch Ziekenhuis Rotterdam - Dijkzigt waar in nauwe samenwerking met de afdeling Cardiologie het in dit proefschrift beschreven onderzoek werd uitgevoerd onder begeleiding van Carla Baan, Aggie Balk en Willem Weimar.

Dankwoord

Omdat bijna iedereen die dit boekje in handen krijgt dit dankwoord leest, is het de kunst om niemand over te slaan die een belangrijke bijdrage heeft geleverd aan de tot stand koming van mijn proefschrift.

Allereerst wil ik mijn promotor Willem Weimar en mijn copromotoren Carla Baan en Aggie Balk bedanken. Willem bedankt voor de mogelijkheid om dit promotie-onderzoek uit te voeren en voor de stimulans die ik af en toe nodig had om mijn onderzoeksresultaten eens vanuit een andere hoek te bekijken. Carla bedankt voor het vinden van lichtpuntjes als de resultaten tegen vielen en voor het stellen van die vervelende 'waarom'-vragen waardoor ik werd aangezet om weer een stapje verder te denken. Aggie bedankt voor de moeite om (zelfs in de vakantie) de echo-stukken serieus door te nemen om de puntjes op de 'i' te krijgen.

Alhoewel ik geen 'eigen' analist had, waren er gelukkig altijd analisten bereid om een stukje van het lab-werk op zich te nemen. Wendy en Lisette bedankt voor het uitvoeren van een deel van het competitieve PCR werk. Sandra en Ronella dank ik voor het uitvoeren van de immunohistochemische kleuringen en voor de hulp bij het fotograferen van de coupes. Mariska bedankt voor het opzetten van de FLIP real-time PCR en het doormeten van FLIP in een groot aantal biopten. Elly Hagman van de afdeling Echocardiografie dank ik voor het uitvoeren van de tijdrovende analyses van alle opgenomen echo's. Roel de Weger en Dick van Wichen van de afdeling Pathologie uit Utrecht wil ik bedanken voor de prettige samenwerking gedurende het afgelopen jaar. Daarnaast dank aan Jolanda, Edwin, Suzan, en Judith van de afdeling Virologie omdat zij altijd met raad en daad klaarstonden bij problemen met de RNA isolaties en de PCR. Ook wil ik Bert Niesters, Lex Maat, Pieter Zondervan, en Teun van Gelder bedanken voor hun bijdrage als mede-auteurs van de artikelen. Verder dank ik ook Willy, Patricia en Annemarie van de D-vleugel en Baukje van Cardiologie voor het regelen van afspraken en handtekeningen.

Speciaal wil ik Cecile bedanken voor de gezelligheid en steun ('samen in hetzelfde schuitje') tijdens congressen en sinds kort ook op de kamer. Ook wil ik haar en mijn beste vriendin Kristine bedanken voor de bereidheid om mij (letterlijk en figuurlijk) ter zijde te staan als paranimf tijdens de promotieplechtigheid. Verder wil ik alle anderen

bedanken die gedurende de afgelopen vijf jaar hebben bijgedragen aan de gezellige werksfeer op en rond het transplantatielab. Nicole, Barbara, Len, Cees, Iza, Franciska, Annemiek, Saskia, Wenda, Thea, Joke, Francine, Petros, Hien, Dennis, en Debora, maar ook Paula, Chris, Marij, Frans, Maud, Arnout, en Muge die inmiddels elders werkzaam zijn: bedankt.

Tenslotte wil ik ook iedereen in mijn directe omgeving bedanken voor de interesse in de voortgang van mijn promotie-onderzoek en dan met name natuurlijk dank aan Tjeerd.