Reverse remodeling of the extracellular matrix in heart failure after left ventricular mechanical support

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Reverse remodeling van de extracellulaire matrix in hartfalen na mechanische ondersteuning van de linker ventrikel (met een samenvatting in het Nederlands)

Proefschrift

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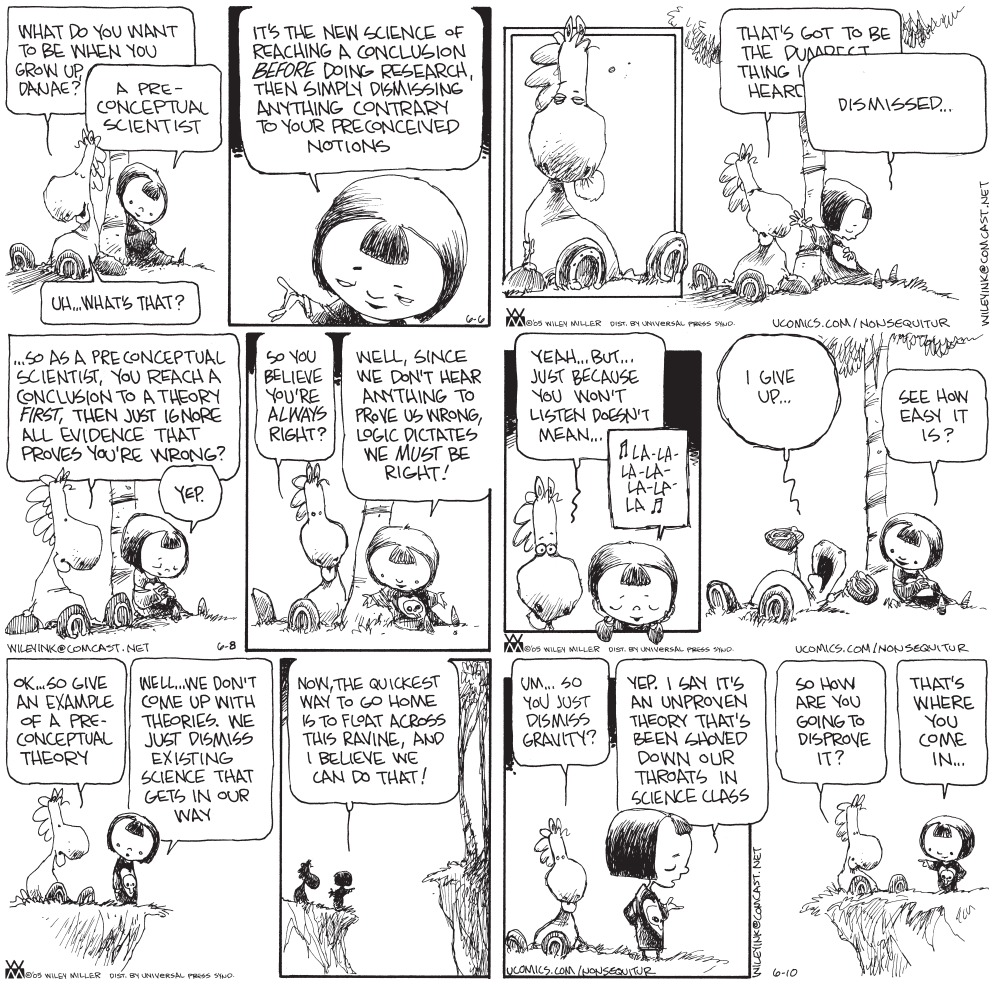

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List of abbreviations

ACE	angiotensin converting enzyme
ADAM	a disintegrin and metalloproteinase
ANG (II)	angiotensin two
BM	basement membrane
BNP	brain natriuretic peptide
cGMP	cyclic guanine monophosphate
DAB	diaminobenzidine
DCM	dilated cardiomyopathy
ECM	extracellular matrix
GM-CSF	granulocyte macrophage colony stimulating factor
GZ	gel zymography
HF	heart failure
HTx	heart transplantation
IHC	immunohistochemistry
IHD	ischemic heart disease
HRP	horseradish peroxidase
IABP	intra aortic balloon pump
ISH	in situ hybridisation
ISZ	in situ zymography
kDa	kilo dalton
LV	left ventricle
LVAD	left ventricular assist device
LVEF	left ventricular ejection fraction
MAP	mean arterial pressure
MMP	matrix metalloproteinase
MT-MMP	membrane bound matrix metalloproteinase
mRNA	messenger ribonucleic acid
NFκB	nuclear factor kappa beta
NPRA	natriuretic peptide receptor A
NYHA	New York Heart Association
PBGD	porphobilinogen deaminase
PVR	pulmonary vascular pressure
Q-PCR	quantitative polymerase chain reaction
RAAS	renin angiotensin aldosteron system
REMATCH	randomized evaluation of mechanical assistance for the treatment of congestive heart failure
TEM	transmission electron microscopy
TIMP	tissue inhibitor of matrix metalloproteinase
TNF-α	tumor necrosis factor-alpha
TNFR-1	tumor necrosis factor receptor-1
TNFR-2	tumor necrosis factor receptor-2
VO_2	oxygen consumption

1

General Introduction

Heart failure (HF) is a growing worldwide public health problem and is one of the leading causes of hospitalization of elderly patients. As much as one third of the patients dies within the first year of diagnosis, and half of the patients does not survive five years [1]. HF is a clinical syndrome in which the heart is unable to deliver sufficient blood for a normal body function. The most common cause of HF is left ventricular dysfunction due to coronary artery disease [2]. Other important causes of heart failure are hypertension, valvular disorders, and cardiomyopathies. The increase in left ventricular (LV) chamber volume (dilatation) and myocardial mass are important consequences of LV dysfunction. Although dilatation of the left ventricle is a compensatory process which maintains cardiac output initial, this is however, associated with the onset of progressive LV dysfunction, and with decreased survival in the long term. The process of structural changes of size and shape of the heart, together with myocardial hypertrophy and fibrosis is called "structural ventricular remodeling" [3].

Remodeling of the heart

Remodeling is characterized by structural rearrangement of the architecture of the cardiac ventricular wall. It involves hypertrophy of the cardiomyocytes, fibroblast proliferation, and increased deposition of extracellular matrix (ECM) proteins [4, 5]. Myocardial remodeling is mainly triggered by mechanical stretch, but also other factors such as ischemia and hormonal changes may definitely play an important role. Initially the term remodeling was limited to indicate the changes which occur following myocardial infarction. Currently, the term is used in a much wider sense. Although remodeling does not necessarily define a pathological condition, myocardial remodeling is only used in disease conditions [6]. The result of myocardial remodeling includes progressive worsening of the systolic and diastolic function, development of mitral valve insufficiently, and an increased risk for arrhythmias [7]. Since cardiac remodeling is an important aspect of disease progression, preventing or reversing the process of remodeling would be an important therapeutically target[7]. This process requires a profound knowledge of the process of remodeling and is subject of this thesis.

The Extracellular matrix (ECM)

Fibrillar collagens

In addition to cardiomyocytes, the myocardium consist of many other cellular components, like fibroblasts, endothelial cells, mast cells, macrophages and the extracellular matrix [8, 9]. The ECM constitutes a structural network which comprises proteins like collagens and elastin, specialized proteins like laminin, fibronectin and proteoglycans. This network forms a continuum throughout the heart. It connects cardiomyocytes to cardiomyocytes, cardiomyocytes to capillaries, and cardiomyocytes to large collagen fibers. Furthermore, it

forms fine connections between large collagen fibers[8-10]. The fibrillar collagens in the heart consist of collagen type I and type III. Type I collagen is important for tensile strength, whereas type III maintains structural integrity of the heart [9]. Even a small increase in the amount of collagen results in increased systolic and diastolic stiffness, while decreased levels of collagen lead to myocardial dilatation [8, 10]. Type I and type III collagens are present in the form of long polypeptide chains called α chains which associate to form a triple helix. These helices aggregate to form fibrils. This fibril formation takes place in the extracellular space, and involves cleavage of terminal pro-collagen peptides by specific enzymes [11]. Structurally, there are three components of the fibrillary collagen network (figure 1) [10, 12].

1) The epimysial collagens. They cover the entire myocardium.

2) The perimysial collagens. These are bundles of collagens which connect the epimysium to the endomysium and surround groups of myocytes. The perimysial strands provide lateral connections between myocyte bundles and thereby prevent slippage of cardiac muscle.

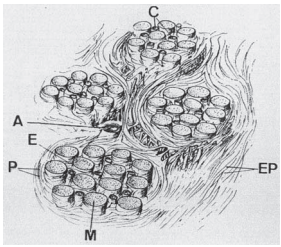

Figure 1. A schematic representation of the myocardial collagen network. EP-epimysium; P-perimysium; E-endomysium; M myocyte, A-arteriole; C capillairies. (Adapted from 10)

3) The endomysial collagens, also called struts. These collagens are found around individual myocytes [10, 13, 14]. They penetrate into the basement membrane and the sarcolemma of the cell, and communicate with the cytoskeleton that supports the actin-myosin contractile protein apparatus [15]. Fibrillar collagens are resistant to cleavage by most proteinases, due to their rigid and compact structure. Among the few proteinases that do have the capability to cleave collagens are some of the matrix metalloproteinases and cysteine proteases, as well as a couple of serine proteases.

Basement membrane

Myocytes are surrounded by a basement membrane which consists of type IV collagen, heparin sulfate proteoglycans, entactin and laminin (figure 2) [11, 16, 17]. All components of the basement membrane are synthesized by the cardiomyocytes. The basement membrane is attached both to the sarcolemma and to the collagens in the ECM (figure 2). The connection of the myocytes with the basement membrane is a major determinant maintaining their shape and positional integrity within the myocardium. Changes in myocyte-

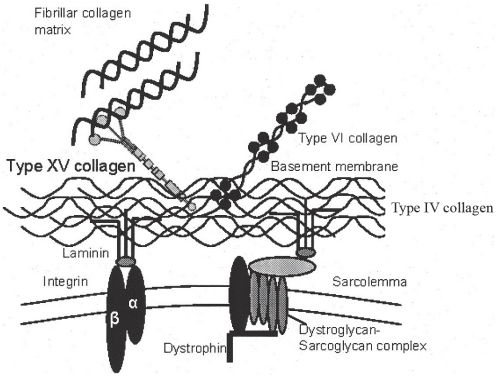

Figure 2. Structure of the basement membrane (BM). The BM is linked to the sarcolemma by the sarcoglycan complex and via laminin-integrin binding. Furthermore the BM is linked to the extracellular matrix by type XV and type VI collagens Modified from Eklund et al. 2000, Sasaki et al. 2000 and Towbin & Bowles 2001 et al.

Figure 3. The structure of the basement membrane which is formed by the interaction between type IV collagen, laminin, entactin and proteoglycans. (Adapted from 21)

basement membrane attachment may cause ventricular dilatation and remodeling and may therefore contribute to ventricular pump dysfunction [18]. Type IV collagen differs from the interstitial collagens by the presence of globular domains interspersed within the triple-helical segments[19]. This unique feature gives type IV collagen the flexibility to assemble into a sheet-like network (Figure 3). Type IV collagen in the BM links the sarcolemma of individual myocytes to the surrounding ECM and interstitial cells [8] and is important in the transmission of force during diastole and systole. Type IV collagen is composed of three α chains. Currently, six types of these α chains (designated as 1 through 6) have been identified. The most abundant form of type IV collagen is a hetero-trimer of α1(IV)2α2(IV) which is present in all basement membranes [20]. Laminin, another important component of the basement membrane, is composed of three different chains which occur as various types: five α chains, threeβ chains and three γ chains[21, 22]. Not all possible combinations of these three chains occur: 12 distinct isoforms have been isolated. The primary role of laminin is the interaction between cardiomyocytes and the basement membrane [11].

Enzymes involved in remodelling of the ECM

Matrix metalloproteinases

The matrix metalloproteinases (MMP) are a family of zinc-dependent endo-proteases which are responsible for the degradation of the ECM and therefore involved in several cardiovascular disease processes. These include LV remodeling as it occurs following pressure and/or volume overload [23-26], and progression of congestive heart failure progression [27]. At this time, over 25 distinct MMP have been characterized [27, 28]. They all share the same structural domains but differ in their substrate specificity, inducibility, and cellular sources (Figure 4). Once the MMP mRNA is expressed, the enzyme is translated and very rapidly secreted in the form of a proenzyme (zymogen), which needs to be activated before it can degrade the components of the ECM. When activated, MMPs are able to cleave at the unique Gly-Leu or Gly-Ile sites in the native collagen triple helix chains. These sites are located at ¾ from the N-terminal, generating ¾ (TCA) and ¼ (TCB) collagen fragments (Figure 5). Thermal degradation and loss of stability rapidly results in the loss of their triple helix conformation. The resulting single α-chains (gelatins) can be further degraded into single amino acids and oligopeptides [8, 29]. Activation of the zymogen occurs by cleavage of a pro-peptide domain. This results in a conformational change which exposes the catalytic

domain to the extracellular matrix[8, 27]. Activation of the MMP within the extracellular matrix can occur through many mechanisms. Plasmin, produced from its plasminogen precursor, can convert both pro-collagenase and pro-stromelysin to their active forms. The membrane-bound matrix metalloproteinases and also cytokines such as TNFα can activate several MMPs [30, 31]. MMP are divided in four major groups (Figure 4): The interstitial collagenases (like MMP-1, MMP-8 and MMP-13), the gelatinases (MMP-2 and MMP-9), stromelysins (MMP-3) and the membrane bound form (MT-MMP)[8, 27, 32].

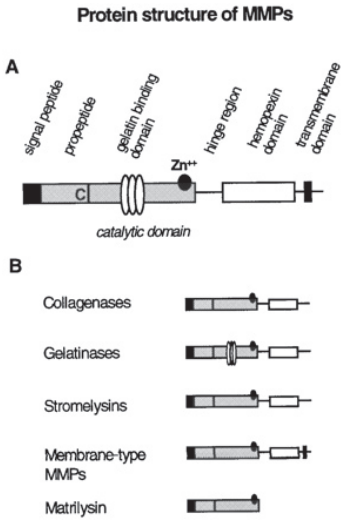

Figure 4. Basic domain structure of MMPs. A. MMP contain at least 3 homologous protein domains: the signal peptide, the propeptide domain, and the catalytic domain. B, Structural differences between the various classes of MMPs. (Adapted from 32)

Collagenases

The family of the collagenases consists of three members: Interstitial collagenase (MMP-1), neutrophil collagenase (MMP-8) and collagenase 3 (MMP-13). Collagenases are of essential importance for the remodeling of collagen networks, because they are the sole enzymes capable of degrading collagens type I, II and III. All three MMPs catalyse cleavage exclusively at a single locus (see above), producing characteristic three quarters/one quarter digestion products. these are soluble, and susceptible to further degradation by gelatinases and stromelysins [33, 34]

Gelatinases

The gelatinase family consist of two members. The 72 kDa type IV collagenase or gelatinase A (MMP-2) [35] and the 95 kDa gelatinase B (MMP-9) [36]. The gelatinases are the only MMP having an insert in their catalytic domain consisting of three tandem repeats of fibronectin type II sequences that are thought to confer gelatin binding properties[37, 38]. Furthermore, the gelatinases are able to degrade elastin, fibronectin, type IV, V, and VII collagens, and gelatins. Differences between the two gelatinases are demonstrated by their selective association with the tissue inhibitors of MMP, the so called TIMP. MMP-2 binds to TIMP-2, whereas MMP-9 binds to TIMP-1 [36, 38, 39].

Stromelysins

Stromelysins, such as MMP-3, are produced by a variety of cell types and can degrade a large number of extracellular substrates such as type III, IV and V collagens, laminins, fibronectin and proteoclycans [40]. In addition, MMP-3 can also proteolytically activate other MMP. [41].

Matrilysin

Matrilysin, MMP-7, is similar to stromelysin in its substrate specificity, but similar to the

interstitial collagens in the crystal structure of its catalytic domain. Matrilysin is unique because it lacks the carboxy terminal segments present in other MMPs.

Membrane type metalloproteinases (MT-MMP)

The MT-MMPs are the most recently characterized MMP subfamily. At this moment, 6 members are known [42, 43]: MT1-MMP (MMP-14), MT2-MMP (MMP-15), MT3-MMP (MMP-16) and MT4-MMP (MMP-17) MT5-MMP (MMP-24), MT6-MMP (MMP-25). The MT-MMPs are present in the normal human left ventricle and are an important system for activation of MMPs within the myocardium. It has been demonstrated that TNFα can upregulate the levels of certain MT-MMPs [44].

Tissue inhibitor of metalloproteinases (TIMPs)

The activity of MMP is tightly controlled by tissue inhibitors of metalloproteinases (TIMPs) [45]. TIMPs are able to inhibit MMP activity by binding to the active enzymes in a 1:1 stoichiometric ratio [46]. To date four TIMPs have been identified. TIMP-1 binds with high affinity to activated MMP; TIMP-2 forms a complex with MT-MMP and this complex enhances the activation of proMMP-2 [27]. TIMP-3 is ECM-bound, and inhibits MT-MMP[47]. In addition to its function as an inhibitor of MMPs, TIMP-3 has been reported to inhibit the shedding of cell surface-anchored molecules such as tumor necrosis factor-α receptor, by other enzymes [48]. TIMP- 4 appears to have a predominant distribution within the myocardium and inhibits the activity of MMP-1, MMP-2, MMP-3, MMP-7 and MMP-9.

MMPs and TIMPs in heart failure

Most of the MMP are constitutively expressed in the heart, but the level of MMP expression is highly inducible. MMP expression depends on the response to multiple stimuli, including mechanical stretch, growth factors, cytokines and other bioactive peptides [49]. The majority of the MMP are present in the ECM in a latent, inactive pro-form. In the myocardium, fibroblasts, mast cells and cardiomyocytes are believed to synthesize and secrete the MMP[50-52]. In HF, MMP expression and activity are upregulated. This suggests that matrix remodeling by MMP is a common response of the myocardium to tissue injury [27, 53-55]. All four TIMPs are also constitutively expressed in the heart and are in balance with the MMP's. In end-stage heart failure a MMP/TIMP imbalance has been observed [56, 57]. Although MMPs have an important role in extracellular matrix remodeling, there are several studies suggesting that also certain Cathepsins may contribute to the cleavage of type I collagen in the ECM.

Cathepsins

For historical reasons, intracellular proteases were named cathepsins. All known lysosomal cysteine proteases are cathepsins, but not all cathepsins are lysosomal or cysteine proteases. Cathepsin A and G are serine proteases and cathepsin D and E are aspartic proteases; cathepsin E and G are not lysosomal proteases. Another group of cathepsins are the papain-like

peptidases. There are 11 papain like peptidases known at this moment: cathepsin B, C, F, H, L, K, O, S, V, X and W [58, 59]. Cathepsins are synthesized as inactive preproenzymes and are glycosylated post-translationally. They are directed towards the lysosomal compartment using cellular mannose-6- phosphate receptors. Seven of the papain-like lysosomal peptidases, the cathepsins B,C,F,H,L,O and Z are ubiquitously expressed in human tissues including the myocardium. Cathepsin K is mainly found in osteoclasts [60, 61] but recent studies showed that Cathepsin K is also expressed in the heart [59, 62]. Because Cathepsin K is further analysed in this thesis, Cathepsin K will be described in more detail.

Cathepsin K

Cathepsin K is a cysteine proteinase which plays an important role in bone resorption [63]. The enzyme is highly expressed in osteoclasts and ovaries and is upregulated at sites of inflammation. To a lesser degree it is also expressed in heart, lung, skeletal muscle, colon, and placenta [64, 65]. Cathepsin K has strong collagenase, elastase and gelatinase activity and, therefore, also possesses the ability to induce remodeling of the extracellular matrix. It may act either on its own, or in concert with other collagenolytic and gelatinolytic enzymes. Cathepsin K cleaves the collagen molecules both into the telopeptides and at multiple sites within the native triple helix (figure 5), thereby generating fragments of various sizes [63]. The role of cathepsin K in extracellular matrix remodelling in HF has not been studied.

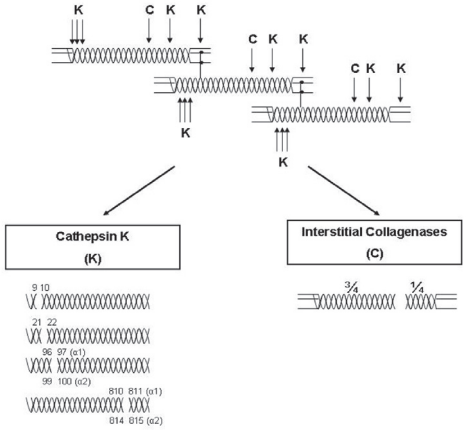

Figure 5. Interstitial collagenases (c), cleave type I collagen within the native triple helix at a single site generating the ¾ N-terminal and ¼ C-terminal fragments. Cathepsin K (k) cleave type I collagen both in the telopeptides and at multiple sites within the native triple helix, generating fragments of various sites. (Adapted from 63).

Regulatory mediators in remodeling

LV remodeling is a dynamic process that occurs in response to pathologic stimuli. This response may be viewed as an attempt to maintain cardiac output and normalize wall tension [66]. A variety of primary compensatory mechanisms is operational in HF [67]; They include the sympathic nervous system, the renin-angiotensin-aldosteron-system (RAAS)[68], the natriuretic peptide, and cytokine systems [69, 70]. The sympathic nervous is not discussed in further detail.

Renin-angiotensin-aldosteron system (RAAS)

The renin-angiotensin system plays an important role in regulating the blood volume, arterial pressure, and cardiac and vascular function. Renin is a proteolytic enzyme that is synthesized in the juxtaglomerular cells of the kidneys. It converts angiotensinogen, produced by the liver, to angiotensin I. Angiotensin converting enzyme (ACE), produced by the lungs, is necessary for the generation of the biologically active component, angiotensin II (AngII)[71]. The haemodynamic effect of increased RAAS activation is vasoconstriction in an attempt to conserve central volume. AngII and aldosterone (which acts on the tubules in the kidney to re-absorp more sodium and water from the kidneys) stimulate collagen synthesis while AngII additionally suppresses the activity of MMP-1, the key degradation enzyme of type I collagen, that synergistically leads to progressive collagen accumulation within the myocardial interstitium [72, 73]. Therapeutic manipulation of this pathway is a very important means of treating hypertension and heart failure [74]. Interruption of the RAAS with ACE inhibitors and AngII receptor blockers reduces cardiovascular morbidity and mortality in patients with heart failure [75]. In addition,RAAS activation is opposed by increased levels of natriuretic peptides. The natriuretic hormones regulating fluid balance in addition to and opposing the action of the RAAS. However, in patients with heart failure there is an imbalance of this circulatory control towards vasoconstriction [76].

The natriuretic peptide system

The natriuretic peptide system consist of atrial natriuretic factor (ANP), brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP). They are produced by several tissues, but mainly in the artia of the heart (ANP) the ventricles (BNP) and the vascular endothelium (CNP). ANP and BNP were shown to be important modulaters of the renin-angiotensin-aldosteron system and act as counteracting hormones for a system that tend to increase blood pressure [77]. BNP is further analysed in this thesis and therefore described in more detail.

BNP

BNP is released as preproBNP, which is cleaved into pro-BNP, and subsequently into the active hormone BNP [78]. BNP is known to have natriuretic, diuretic and vasorelaxant properties and has antagonistic effects on the renin-angiotensin-aldosteron system [79]. BNP plasma levels are increased in patients with heart failure and correlate with left ventricular ejection fraction [80]. BNP plasma levels are therefore used as diagnostic and prognostic markers in HF [80-82]. During the early phase of acute myocardial infarction, BNP plasma levels are increased as well [83]. This is suggestive of an important additional role for BNP in the process of tissue remodeling. Furthermore, it has been demonstrated that BNP exhibits an anti-fibrotic effect by decreasing collagen synthesis and increasing MMP production [84].

Cytokines

Cytokines are a group of immune response mediators, including the families of interleukins, interferons, chemokines and growth factors. Cytokines are responsible for the intercellular communication between cells involved in immune responses. Most cells of the immune system, including T cells, B cells, NK cells, macrophages, dendritic cells and mast cells, are able to produce one or more cytokines. Many other cell types, including cardiomyocytes can produce cytokines as well [85, 86]. The production of cytokines is inducible; the peptides are rapidly secreted and have a short half-life time. Systemic cytokine concentrations are low: most cytokines act locally, either on the cell of origin or on cells in the immediate neighbourhood. Typically, cytokines can have different effects on different cell types, whereas different cytokines may have similar functions [87]. Although different cytokines belong to multiple categories, the cytokines can be classified into distinct functional classes:

1. Cytokines that mediate innate immunity like IL-1, Tumor Necrosis Factor-α (TNFα), IL-6, and type 1 Interferons. Most of these are also designated as pro-inflammatory.
2. Cytokines that regulate the growth, activation and differentiation of lymphocytes, such as IL-2, IL-4, IL-12, IL-15, and Transforming Growth Factor-beta (TGFβ).
3. Chemotactic cytokines such as Il-8, CXCL 13 and MCP.
4. Cytokines that induce proliferation, like CSF, IL3 and IL5[88].

The Production rate of cytokines may be related to certain single nucleotide polymorphisms (SNP), particularly those within the promotor regions of cytokine genes. In this thesis the promotor polymorphisms of IL4, IL6, TGFβ, and TNFα have been investigated in patients undergoing a heart transplantation. The best characterized inflammatory cytokine in congestive heart failure is TNFα and therefore, mainly TNFα will be discussed in this cytokine overview.

TNFα

TNFα belongs to the TNF-ligand family and has multiple biologic activities. TNFα is one of the main pro-inflammatory cytokines and plays a role in initiating and regulating the cytokine cascade (e.g. IL-1 and IL-6) during an inflammatory response. The TNFα structural gene and regulatory regions contain many different polymorphisms and microsatellites[89, 90]. At least three of them are located in the promoter region and seem to be strongly related with the production of TNFα. In all three polymorphisms the common nucleotide “G” is substituted by an “A”. Wilson et al. described that the “A” at position –308 (indicated as TNF2 allele) constitutes a much more powerful transcriptional site than the “G” at that position (indicated as TNF1 allele)[91]. Although there is no known nuclear protein specifically binding at the polymorphic site, these authors suggested that this polymorphism may have a direct effect on the transcriptional activity. They demonstrated a six to sevenfold higher level of transcription from the TNF2 allele in in vitro studies.

Different studies in man and mouse show that over-expression of TNFα may have a role in the development of heart failure. TNFα causes a decrease in cardiac contractility, in combination with cardiomyocyt apoptosis, resulting in a DCM in animal models [92-94]. In patients with heart failure the myocardium itself synthesize TNFα [95]. It is produced by myocardial cells through the nuclear factor kappa B (NFκB) in response to pressure or volume overload, endotoxins and oxidative stimuli [96, 97]. Heamodynamic overload or myocardical stretch provokes TNFα mRNA and protein synthesis [95]. TNFα is capable of modulating cardiovascular functions through a variety of mechanisms, such as left ventricular dysfunction [98] and left ventricular remodeling [99]. Furthermore, TNFα is involved in the production of other cytokines like IL1 and IL6 which enhance TNFα induced myocardial depression and cytotoxity [100, 101]. TNFα acts on two TNF receptors (TNFR): TNFR-1 and TNFR-2. The TNFR are present on all nucleated cells in the heart; they can be 'shed' from the cell membrane and become detectable in the plasma as soluble receptors (sTNFR) [102, 103]. TNFR-1 appears to be the main signaling receptor and is most abundantly expressed. The majority of the effects produced by TNFα act via this receptor [104]; TNFR-2 would rather appear to have a somewhat protective role in the heart [105]. Both receptors retain their ability to bind with TNFα when they are shed from the membrane, and can inhibit at least the cytotoxic activities of TNFα [106]. Antagonizing the actions of TNFα with a recombinant soluble TNF receptor that binds to TNFα and neutralizes it, seems to be a good therapeutical option in patients with heart failure on theoretical grounds, but studies in humans so far have been disappointing [107].

Heart transplantation

When patients develop end-stage heart failure and their cardiac function cannot be improved medically or surgically, heart transplantation can be considered.The first human-to-human heart transplantation (HTx) was performed in December 1967. Although the transplanted heart functioned well, the patient died of pneumonia after eighteen days. Autopsy showed features of mild to moderate acute rejection. Acute rejection or infections were the major causes of death after HTx until, in 1980, cyclosporin was introduced as an immunosuppressive agent. This led to a increase in the number of heart transplantations. To this day, more than 73,000 HTx have been performed worldwide[108] . The current one-year survival of HTx is 80-85%. In the Netherlands, two centers perform HTx: Erasmus Medical Center Rotterdam (Erasmus MC) and the University Medical Center Utrecht (UMC Utrecht). Together they perform approximately 40 HTx annually. Due to the shortage of donor hearts, heart transplantation can only be offered to a highly selective group of patients. Therefore, it is necessary to explore other therapeutic options for patients with end-stage heart failure. One of these is mechanical circulatory support.

Mechanical circulatory support

Over the last two decades, several mechanical circulatory support devices have been developed. They can be used as a bridge to cardiac transplantation, to support recovery, and even as an alternative to heart transplantation: destination therapy. Already in 1969, a totally pneumatically driven artificial "heart" was available to span the time until transplantation [109]. The current generation of devices can be divided into three groupes, intended for short-, intermediate- and long-term duration respectively. They can be additionally categorized as partial left ventricular support, more complete left ventricular support, right ventricular support, and biventricular support. An example of a short-term device is the intra-aortic balloon pump (IABP). Some examples of long-term assist devices are the Thoratec (Thoratec Corp, Pleasanton, CA), the Novacor (World Heart, Ottawa) and the Heartmate (Thoratec Corp, Pleasanton, CA). The Thoratec ventricular assist device can be used for univentricular as well as for biventricular support. The pumps are placed externally on the anterior surface of the abdomen, limiting mobilization of the patient. Intensive anticoagulation is required [110]. The Novacor is an electrically driven, implantable pump, limited to left ventricular support. Newer devices are already available, some more reliable and simple to operate than the above mentioned devices. Some examples are the Heartmate II and the Jarvik 2000 [111]. They are axial-flow devices that provide continuous flow from the left ventricle to the aorta. In the present study, the type HeartMate XVE was used. It will therefore be described in more detail.

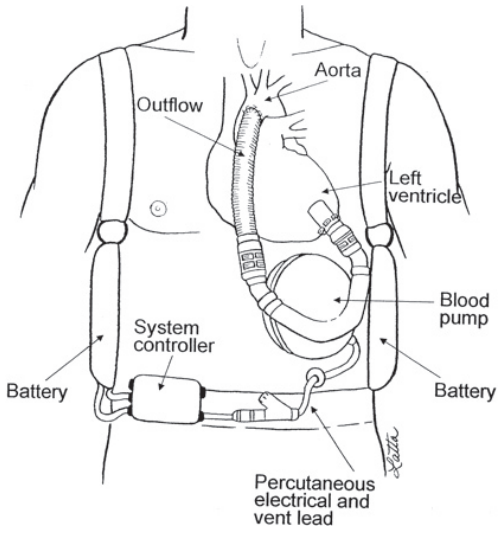

Figure 6. Schematic picture of the HeartMate XVE left ventricular assist device. Source: Prog. Cardiovasc. Nurs © 2004 Le Jacq Communications, Inc

HeartMate XVE

The HeartMate XVE supports the left ventricle and is the main device used in the patients described in this thesis. The HeartMate pump itself is a flattened titanium cylinder about five cm thick and ten cm in diameter. The device weighs about 1,200 grams. The blood pump has two chambers: the blood chamber and the motor chamber. A flexible diaphragm separates these two chambers. The diaphragm moves up and down when pushed by the motor. The inside of the pump has a special textured surfaces. One side is made of titanium, while the other is a textured polyurethane diaphragm. The textured surfaces stimulate the gradual generation of a cellular lining, mimicking

a vascular endothelium. This helps prevent local blood clotting, limiting anticoagulation therapy to a daily gift of aspirin. The device is connected by cannulas, containing porcine valves, between the apex of the left ventricle and the base of the aorta. The HeartMate device is capable of a stroke volume of 83 ml, and of a pumping capacity up to 10 liters per minute. Through the external system controller, the pump is instructed in terms of beat frequency and (fixed rate or auto rate) beat mode, and constantly checked for proper functioning.

The HeartMate left ventricular assist system (LVAD) has supported more than 2,800 patients [112] world wide. The usefulness of the left ventricular assist device (LVAD) as a permanent form of circulatory support has been established in the REMATCH (Randomized Evaluation of Mechanical Assistance for the Treatment of Congestive Heart Failure) trial, particularly for a selected group of patients with end-stage heart failure, who are noneligible for heart transplantation [113, 114]. The survival during device support is about 83% at 1 month and 50% at 1 year [115]. The outcome after heart transplantation in these patients is comparable to that of patients transplanted after having been only supported by inotropic therapy. The major causes of death in these patients are multi organ failure and bleeding complications around the time of surgery. Post implantation, the major limitations of the devices are infections, bleeding, and device mal dysfunction [115].

Considerations for LVAD use.

The use of LVAD as a bridge to heart transplantation does not result in more heart transplantations; the LVAD only supports those patients with the most serious condition and the greatest risk of dying, while awaiting cardiac transplantation on medical therapy. It has been suggested that a LVAD can be used as bridge to recovery of cardiac function [116, 117]. Prolonged mechanical circulatory support of the left ventricle does result in a decrease of cardiac dimension [118-120] and cardiomyocyte size [121-123]. The contractile function of the myocytes improves [124, 125] with a resultant decrease of neuro hormonal activation [126-128]. However, there is only a limited experience with weaning of patients from the device and the feasibility of the entire policy has been seriously questioned [129, 130].

LVAD implantation as a model to extracellular matrix (reverse) remodeling

LVAD implantation offers the possibility for obtaining myocardial tissue of patients with end-stage heart failure, as insertion of the inflow canula in the left ventricle requires the removal of a part of the apex (pre-LVAD). At the time of heart transplantation more myocardial biopsies are taken. In this way human myocardial tissue of patients with end-stage heart failure can be compared with that after assist device implantation. This allows to investigate reverse remodeling of the extracellular matrix over the period of mechanical circulatory support. Our group has previously demonstrated that LVAD support in patients with end stage heart failure leads to improved patient condition [131] and reduction of cardiomyocyte size [123]. This process of reverse remodeling led to dramatic changes in cardiac size. It is therefore hypothesized that the ECM, as a consequence, also adapts to these changes.

Aim of this study.

At this moment LVAD's are still primarily used as a bridge to heart transplantation. Ventricular unloading provided by the LVAD may be effective to induce reverse remodeling. Echocardiographic and hemodynamic studies have shown that unloading of the heart is associated with improved ventricular function, with reduction of markers of neuro-endocrine activation and with a reduction in inflammatory markers during LVAD support [122, 127, 128, 132-134]. Furthermore, there is evidence for reverse remodeling both at the level of the cardiomyocyte and the structure and function of the heart [121]. Conflicting data exist to regarding normalization of the extracellular matrix after LVAD support.

The aim of the study described in this thesis is first to investigate the changes in the ECM during LVAD support, in order to better understand the role of the ECM in HF and secondly, to answer the question whether LVAD support contributes to the recovery (reverse remodeling) of the LV myocardium. The results might indicate whether weaning of the LVAD would yet become a feasible option in the near future.

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Cytokine gene polymorphism in heart transplantation: a review

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Abstract

Cardiovascular disease is the most common cause of death in today's society. Heart transplantation is the only available treatment for patients suffering from end-stage congestive heart failure (CHF). Causes underlying the development of CHF are still unknown, but it has been suggested that pro-inflammatory cytokines play an important role. In the context of transplantation, pro-inflammatory T-helper 1 cytokines like TNF-α, IL-2 and that mediate the cellular immune response are believed to be involved in acute graft rejection. On the other hand, Th2 cytokines, like IL-4, IL-6 and IL-10, might induce tolerance, by down-regulating the Thl response and cytokine production. Cytokine release by macrophages, lymphocytes and other cell types in the microenvironment of the graft, especially the balance between Thl and Th2 is thought to be critical for the incidence of acute rejection. Production of cytokines has been shown to be under genetic control of single nucleotide polymorphisms (SNP) in mainly the promoter regions of cytokine genes. Genotypes of these SNP were shown to mediate differential production of these cytokines, leading to a wide variety of cytokine patterns among individuals. Since levels of these cytokines might affect the Thl/Th2 balance, genotypes might be related to acute allograft rejection. Many studies have shown an association between cytokine gene polymorphisms and the development of several infections, allergies and autoimmune diseases. Also, associations between SNP in different cytokine genes and transplant rejection has been extensively studied. In our lab, SNP in the genes of TNF-α, TGF-β, IL-4 and IL-10 were studied in a panel of 72 cardiac transplant patients and 61 of their donors. These data are discussed in the context of literature data.

Introduction

Heart failure is the most important cause of death in today's society. Heart failure can be divided into two main categories: ischemic heart disease and cardiomyopathies [1]. The largest cause of cardiac mortality and morbidity is caused by ischemic (coronary) heart disease, mostly due to atherosclerosis [1, 2]. Cardiomyopathies are diseases of the heart muscle, of which the underlying cause is largely unknown. Recent studies have identified the importance of biologically active molecules such as pro-inflammatory cytokines (for example IL-1, IL-6 and TNF-α) in disease progression in heart failure. When expressed at sufficiently high levels, these cytokines may mimic certain aspects of heart failure. Examples are progressive ventricular dysfunction and left ventricular remodeling [3, 4]. Significant correlations between elevated levels of TNF-α and IL-6 and disease severity were identified [5, 6]. Until this day, the only treatment for end-stage heart disease is heart transplantation (HTX). The most common causes of mortality in the first 12 months after transplantation are infection (23%) and the occurrence of acute rejection episodes despite the immunosuppressive regimen (19%) [7, 8] Acute rejection episodes are diagnosed by histopathological examination of endomyocardial biopsies (EMB) and graded according to ISHLT grading system, based on the infiltrate of mononuclear cells and cardiomyocyte damage [9]. Cytokine release by T-lymphocytes, macrophages and other cell types in the microenvironment of a transplanted graft is critical in the development of acute rejection. Pro-inflammatory cytokines of the Thl subtype, such as TNF-α, and IL-2, have been implicated in incidence of acute rejection [10]. It has been suggested that cytokines of the Th2 subtype, like IL-4, IL-10 and TGF-β, can play a role in the development of tolerance. Local secretion of Th2 cytokines at a site of inflammation leads to down regulation of Thl cytokine production [11]. It has been postulated, that, in such a way, even a complete Th2 "cytokine field" might be created [12]. Different cell types present in the microenvironment of the heart (donor cells, for example macrophages, fibroblasts, cardiomyocytes and stromal elements), as well as the ones arriving there from the circulation (recipient cells like lymphocytes, monocytes, dendritic cells and natural killer cells) are influenced by this local cytokine field. Moreover, these local players interact with each other and start participating in this Th2 cytokine field by which development of acute rejection is suppressed. According to this model, this Th2 cytokine field might slowly spread even wider, through recirculation of the participating cells, until the entire body tends to a Th2 environment. Emergence and maintenance of a Thl cytokine field in the microenvironment of the heart might promote the incidence of acute rejection. So, especially the Thl/Th2 cytokine balance is of major importance for the incidence of acute rejection. It has been shown that the production level of several of these cytokines is under genetic control through single nucleotide polymorphism (SNP) in the genes encoding these cytokines. The role of cytokine gene polymorphism in transplantation has been studied extensively. In this review, SNP in the genes of TNF-α, IL-6, TGF-β, IL-4 and IL-10 in the context of graft rejection will

be discussed; both our own results (summarized in table 2) as well as studies published over the last 3 years (summarized in table 1 and 3). In this review we will focus mainly on the promoter gene polymorphisms, since these might be localized in binding regions of transcription factors. Therefore, these SNP could influence transcription and as a consequence the level of cytokine production.

Tumor Necrosis Factor-α

Biological activities

TNF-α is a Thl cytokine with a wide range of pro-inflammatory activities. It is mainly produced by macrophages, but also activated T-cells, NK cells, granulocytes, fibroblasts, epithelial cells and even cardiomyocytes are able to produce TNF-α [13-16]. TNF expression is induced by bacterial LPS, mitogens, viruses, other cytokines and is regulated both at transcriptional and posttranscriptional levels [17]. TNF-α production at high levels induces rolling of leukocytes, expression of cellular adhesion molecules and up-regulation of the expression of MHC molecules [11, 18].

Transplantation

Several studies have shown that TNF-α is increased in the myocardium and plasma of patients with congestive heart failure. [19-21]. The infusion of anti-TNF or soluble receptors for TNFα blocks the action of TNFα on the heart and improves the depressed ventricular function [18, 22, 23]. Raised serum levels of TNFα have also been seen in transplant patients during episodes of acute cellular rejection [24]. Localization of the mRNA transcripts and the protein within the rejection infiltrate provided supporting evidence for the role of TNF-α in rejection [24].

Genetic organization and SNP

The gene encoding TNF-α is localized on chromosome 6p21.3 in the MHC class III region, centromeric of HLA- B and telomeric of HLA- DR [25]. There is a strong linkage between HLA alleles and production levels of TNF-α. HLA- Al, - B8, - DR3 expression are associated with high levels of TNF-α whereas HLA- DR4 and HLA- DR6 is correlated with low levels of TNF-α [17, 26, 27]. Within the normal population, there is considerable variation in the TNF-α producing capacity [28]. Several SNP in the TNF-α locus have been identified and were found to play an important role in the production of TNF-α. At least two of them are localized in the promoter region of the TNF-α gene and comprise substitutions from a G to an A at positions -308 and -238 (table 1) [17, 29]. In this review we will focus on the promoter gene polymorphism at position -308. In our study population, only 6 individuals carried both alleles of the SNP at -238 and in most other studies only the -308 polymorphism was studied

as well. Several studies using reporter gene assays or in vitro stimulation assays have demonstrated an increase in the level of TNF-α gene transcription, and a high production of TNF protein when the TNF2 allele (A instead of a G) is present [30, 31]. Various studies indicated a possible relation between acute graft rejection and promoter polymorphisms. Azzawi et al [32]described a strong correlation between the TNF2 allele position -308 (-308*A) and death due to acute cellular rejection. In addition, Awad et al [33] found an association between TNF1 (-308*G, low TNF production) and high IL-10 production, and a low number of acute rejection episodes after pediatric heart transplantation (HTX). In contrast with these studies Jacksen et al [34] did not find any correlation between genotype of TNFα and acute rejection in kidney, heart and lung recipients. In accordance with these results, no correlation between the TNF-α genotype at -308 and the number or severity of rejection episodes after transplantation was observed in our study panel of 72 HTX patients. However, we did find a trend between acute transplant rejection and the high TNF producer -308*A allele in the donor heart. This was also observed by Gandhi et al [35]. These variable results were also described for renal transplantation patients [36-40]. Several groups observed a correlation between TNF2 and rejection, but other groups did not. Several explanations can be given for this. First, Kroeger et al demonstrated that the -308 polymorphism affects TNF transcription both in a cell type dependent and in a stimulus specific manner [41]. Secondly, it's known that immune suppressive regimens interrupt 5'regulation of TNF- a transcription in T-cells and macrophages and possibly has a negate effect of the -308 polymorphism [39]. In conclusion, TNF-α is a potent cytokine, which plays an important role in inflammation and contributes to the development of different diseases. Although the TNF2 allele has been associated with high levels of TNF-α [30, 31], further study are necessary to establish the exact relationship between TNFα gene polymorphisms and acute rejection in transplant recipients. Certainly, the data do not warrant the conclusion that TNFα gene polymorphism is a single decisive trait in heart transplant rejection. Our studies as well as those of Gandhi et al [35] do suggest that the donor SNP genotype is related to acute rejection. This relation should be studied in more detail, since it has been shown that the heart muscle is indeed able to produce its own TNF. Therefore, it is imaginable that TNF-α SNP genotype of the donor heart rather than the patients genotype is associated with acute rejection, by regulating the patients lymphocytes in the rejection reaction.

Interleukin-4

Biological activities

Interleukin-4 (IL-4) is a cytokine of the T-helper 2 (Th2) subtype, which is mainly produced by activated T-cells and mast cells. It is a pleiotropic cytokine that affects cells of multiple lineages [42, 43]. IL-4 is an important mediator of Th2- immune responses. In the induction

phase of the immune response, IL-4 is important for development of $CD4^+$ T cells into Th2 cells. IL-4 also stimulates growth and differentiation of Th2 lymphocytes and mast cells. IL-4 down-regulates the Thl activity and it affects different cell types, a.o. by blocking macrophage activation [44]and inhibiting the production of Thl cytokines like IL-1, TNF-α and IFN-γ in monocytes and activated T-cells [45, 46].

Transplantation

Several studies revealed beneficial effects of IL-4 on graft survival after organ transplantation. Studies in our lab showed that a high frequency of IL-4 producing helper T-lymphocytes (HTL) is indeed related to a reduced incidence of acute rejection in patients after heart transplantation (HTX) [47], and IL-4 mRNA was detected in EMB from HTX patients without rejection or with low rejection grades [48, 49]. Likewise, studies in knock- out mice showed that IL-4 was essential for induction of long-term cardiac allograft survival [50]and IL-4 therapy prolonged cardiac graft survival in rats [51].

Genetic organization and SNP

The human IL-4 gene has been mapped to chromosome 5, where it localizes on the long arm on q31-l and bands together with other cytokine genes including IL-3, IL-5, IL-9, IL-13 and GM-CSF. The IL-4 gene is composed of four exons and three introns, and encodes a mature 129 amino acid glycoprotein [42, 43]. Several polymorphisms have been identified in both the promoter region and in the IL-4 gene itself. In the promoter region, three single nucleotide polymorphisms (SNP) have been described. At position -590 (C to a T), -285 (“C” to a T) and -81 (A to a G) from the transcription start site [52, 53]. The -590*T- allele increases the strength of the IL-4 promoter relative to the -590*C- allele, as measured in a luciferase reporter assay [52]. The polymorphic variants -285*T and -81*G give also rise to differences in promoter strength, mediating overexpression of the IL-4 gene [53]. In our study, we did not detect these polymorphic variants. Studies by Marshall et al [37, 54] revealed no associations between the -590 SNP genotypes and acute rejection after renal transplantation. Our studies indicated no association between patient -590 genotype and acute rejection after cardiac transplantation either. In contrast to this study, a study by Poole et al [55] revealed a protective effect of the high IL-4 producing -590*T allele in the donor on renal graft survival. In our studies a protective effect of-590*T positive genotype in the donor heart on graft survival was identified [56]. Patients who acquired a donor heart with the -590*T- positive genotype suffered significantly less from rejection, especially when they were -590*T negative themselves. This raises the question of which are the potential candidate IL-4 producing cell types within the donor heart, responsible for the protective effect exerted by donor genotype. Mast cells are an abundant source of IL-4. Mast cells are present in the human heart, and have been isolated and purified [57, 58]. Human heart mast
cells have been demonstrated in sections of EMB from HTX patients (unpublished

observation). Since IL-4 has been shown to be involved in initiation of wound healing [59, 60], it might be induced in the heart shortly after the transplantation to initiate repair of operation related and reperfusion damage. This locally produced IL-4 might then be responsible for the local field cytokine production leading to a reduced rejection infiltrate.

Interleukin-6

Biological activities

Interleukin-6 (IL-6), a cytokine of approximately 26 kD, was identified as a T cell derived cytokine promoting the terminal maturation of activated B cells to antibody producing cells [61]. By now, IL-6 has been recognized as a multi functional cytokine, produced by different cell types in response to IL-1 and TNF-α, which can have both favorable and unfavorable effects on human health [61]. Dysregulation of IL-6 expression is linked to the occurrence of a.o. diseases like multiple myeloma and rheumatoid arthritis [62].

Transplantation

High levels of IL-6 can have negative inotropic effects and several studies have shown that IL-6 is up-regulated in patients with depressed left ventricular ejection fraction, and heart failure [63]. Due to the pro-inflammatory capacity of IL-6, it is thought to play an important role in acute cellular rejection. It was show that the pro-inflammatory cytokines like TNF-α, IL-1 and IL-6 were increased in acute rejection after heart- and renal transplantation [64]. Deng et al, showed that IL-6 was associated with impaired allograft function in the absence of rejection [65].

Genetic organization and SNP

The human IL-6 gene has been mapped to the short arm of chromosome 7 (7p21) [62]. Several SNP have been described in the IL-6 gene. In this review we will focus on the promoter gene polymorphism at position -174, which comprises a substitution from a G to a C. The G allele was described to be linked to high production of IL-6 and the C allele to low production [66]. The IL-6 promoter polymorphism at position -174 lies immediately upstream of a multi responsive element (MRE) [66]. This element has been shown to be important in IL-1 and TNF-α modulated expression of IL-6. Cloning of IL-6 promoter containing the -174 polymorphism and expression in a luciferase reporter assay indicated a two fold increase in transcription associated with the C allele . Kilpinen et al [67] found that neonates with the CC genotype had significantly higher levels of IL-6 in plasma than neonates with the GG genotype. They also described that the genetic regulation of IL-6 production after in vitro stimulation of neonatal cells is correlated with the -174 C polymorphism but in healthy adults, the IL6 levels are not correlated with the -174 polymorphism. [67]. Cox et al [68] demonstrated

that there are ethnic differences in the allelic distribution of IL-6 [68]. They found that white individuals carried a G/C allelic distribution of 65%/35% whereas African-Americans had an allelic distribution of 91%/9%. Fisham et al showed that the single nucleotide change from G to C at -174 in the promoter region resulted in suppression of IL-6 transcription in response to LPS and IL-1 in Hela cells [69]. Furthermore, they showed that the C allele position -174 correlates with Systemic-onset juvenile chronic arthritis. Several groups did not find any correlation between the -174 promoter gene polymorphism in patients or donor and acute cellular rejection after heart transplantation [33] and renal transplantion [35, 37, 39]. None of these groups had a clear explanation for the lack of association between acute rejection and the IL-6 gene polymorphism at position -174. Jackson et al [34] observed a trend towards development of coronary artery disease (CAD) in patients with the low IL-6 genotype after transplantation, compared to patients with the high genotype [34]. From these findings they conclude that

high IL-6 production of the patient has a protective role in transplantation. On the other hand, Marshall et al [54] observed a very strong association between the -174*C allele in the donor kidney and acute rejection in the recipient, which indicates that IL-6 production in the microenvironment of the donor heart could also have a negative influence on graft survival. These contradictory results suggest that the amount of IL-6 produced (influenced by genotype -174 of the IL-6 promoter) is cell type dependent. Moreover, this could explain the findings that the SNP genotype of the donor influences acute rejection in renal recipients. In conclusion, it still remains to be elucidated which cell types are important in the production of IL-6 during rejection and how this IL-6 production is regulated, focusing on the microenvironment of the donor organ.

Interleukin-10

Biological activities

IL-10 is an immune modulating cytokine, which is produced by T-cells, macrophages and B-cells [70]. It plays, among others, a role in autoimmunity, tumor genesis and transplantation tolerance [71]. IL-10 exhibits both immune stimulatory and anti- inflammatory activities [72], and is a representative of Th2 responses [73]. On one hand, it contributes to proliferation, differentiation and antibody production by B-cells [73]. On the other hand, IL-10 exerts an anti-inflammatory activity by inhibiting Thl- type responses and monocyte functions. The cytokine inhibits production of pro- inflammatory cytokines such as IL-1, IL-6, IL-8, TNF-α, GM-CSF and IL-12 by activated monocytes, and of IL-2 and IFN-gamma production by activated T- lymphocytes [70]. The level of IL-10 production therefore, appears to be critical, controlling the balance between inflammatory and humoral responses [74].

Transplantation

IL-10, as a Th2 cytokine, might be involved in induction of tolerance towards a transplanted organ [11]. In murine studies, IL-10 was shown to play a role in promoting graft survival by suppression of T- cell and macrophage responses [75, 76]. The presence of IL-10 mRNA and protein has also been demonstrated in EMB from heart transplant recipients by in situ hybridization and immuno-histochemistry [49, 77].

Genetic organization and SNP

The gene encoding IL-10 has been mapped to chromosome 1 and three single nucleotide polymorphisms (SNP) have been identified in the IL-10 promoter region: they are located at positions -1082 (substitution from a G to an A), -819 (C to an T) and -592 (C to an A) upstream from the transcriptional start site. The polymorphisms at -819 and -592 are linked (CC and TA haplotypes) [71, 74, 78, 79]. The -1082*G allele has been correlated with a high level of IL-10 production by lymphocytes upon stimulation with concanavaline A [79, 80]. In another study, the -1082* A allele was correlated with high IL-10 production by whole blood cultures stimulated with lipopolysaccharide (LPS) as compared to the -1082*G allele (Keysers et al., 1997; published as abstract in the abstract book of the Annual meeting of the Dutch Society of Immunology). This indicates that genetical predisposition to produce IL-10 depends on the cell type. Many association studies have been conducted on the polymorphisms in the promoter region of the IL-10 gene and graft survival after organ transplantation. In cardiac graft recipients, the high IL-10 producing genotype (-1082*G) was shown to protect against acute rejection [11, 33, 74] The low IL-10 producing -1082*A genotype was increased in the rejector group [36], and low in the long-term survivor group [39]in renal graft recipients and correlated with incidence of acute rejection after liver transplantation [80], revealing that high IL-10 production improved graft survival. In accordance with these studies, the high IL-10 producing genotype (-1082*G) was indicative of a better kidney graft survival after 5 years [81]. For kidney graft recipients however, also different associations were reported. The -1082*G genotype was significantly associated with an increase in the incidence of multiple rejection episodes, perhaps by promoting vascular rejection processes [82, 83]. In combination with the high TNF-α producing genotype (-308* A, the TNF2 allele), carriers of the -1082*G allele had a bad prognosis for acute rejection after kidney transplantation [84]. In other studies however, no associations were found between IL-10 promoter SNP and rejection following renal transplantation [37, 54] and liver transplantation [85]. In our cardiac transplant cohort no associations were found between either recipient or donor genotype at -1082, -819 and -592 and rejection. (Allele frequencies are summarized in table 2) [86]. According to studies by Turner et al. [11, 79], we expected to find an association between the high IL-10 producing -1802*G allele and decreased incidence of acute rejection after cardiac transplantation. In conclusion, the association described by different groups between IL-10 SNP and acute rejection after organ transplantation varies immensely. Similar alleles would

seem to be associated with reduction or with increase in rejection or to have no effect at all. This might be due to the test models chosen (human or different animals), different organs studied, and the different immunosuppressive regimens. Besides, the IL-10 production levels associated with SNP genotype, have been shown to vary with cell type, e.g. monocytes or lymphocytes. Variations in cellular composition of the inflammatory infiltrate during acute rejection episodes, might thus offer an explanation for the varying results. To this day, no studies have focused on the role of the microenvironment of the donor organ in IL-10 production; it is quite possible that cell types of donor origin, like macrophages, produce IL-10. This could influence the Thl/Th2 balance in the microenvironment in favor of Th2, down regulating development of acute rejection.

Transforming Growth Factor-ß

Biological activities

Transforming growth factor-β is a multifunctional cytokine that is produced by, and affects, variety of cell types in the human body [87]. TGF-β regulates proliferation and differentiation of cells (for example T and B-lymphocytes), embryonic development, wound healing and angiogenesis [87]. The most important functions of TGF-β 1 in the scope of this review are its immunosuppressive properties (acute rejection). TGF-β is produced by all leucocytes, promotes their differentiation, but inhibits their proliferation and activation [88]. Accordingly, development of Th0 cells into Thl and Th2 is inhibited, while after differentiation, Th2 cells are not subject to TGF-β mediated immune suppression [89]. However, pro-inflammatory Thl cell activation and their cytokine production on the other hand are inhibited by TGF-β [89]. Transplantation In the context of organ transplantation, TGF-β production might correlate with reduced incidence of acute rejection, since it down-regulates Thl responses and Thl cytokine production.

Genetic organization and SNP

Several SNP have been described in both the promoter region and the first exon of the TGF-β gene, which encodes the signaling sequence of the TGF-β protein [90]. At position -800 from the transcription start site a substitution from a G to an A has been described [90], and at -509 in the TGF-β promoter region a substitution from a C to a T. The -509*T allele has been correlated with high plasma TGF-β levels relative to the -509*C allele [91]. In the signaling sequence of the protein, a substitution from a T to a C leads an amino acid substitution from a leucin to proline [91], the codonl0*Leu (Leu10) allele being the “high producer” allele of TGF-β by lymphocytes in vitro [92]. On the other hand, another study indicated that the Codonl0*Pro (Pro10) allele was correlated with with increased levels of TGF-β in plasma, indicating the Leu10 allele as the high TGF-β producing allele [93]. The

fourth SNP comprises a substitution from a G to a C [90], leading to an amino acid change from arginin to proline at codon 25 in signaling sequence. The codon 25*Arg (Arg25) allele has been correlated with higher TGF-β production compared to the codon25*Pro (Pro25) allele by lymphocytes in vitro [92]. In various studies relations between on acute graft rejection and TGF-β SNP were investigated. For kidney transplantation, no association between TGF-β SNP and acute rejection was found [37, 39, 54, 55]. The same applied for TGF-β 1 SNP and acute rejection after liver transplantation [85, 94] and pediatric heart transplantation [33]. In our study group, a total lack of association between SNP in the TGF-β gene and heart failure as well as cardiac graft rejection was observed. Allele frequencies are summarized in table 1. Since no relation with acute rejection was observed, TGF-β and its SNP are probably important in regulating mechanisms underlying chronic rejection in transplantation rather than acute rejection. In conclusion, it must be considered that TGF-β is produced by many cell types in the human body [87], including cells in the transplanted organ. TGF-β production by donor cells might therefore influence acute rejection. This question remains to be elucidated.

Conclusion

Cytokines influence each other's function and production. Single nucleotide polymorphism in the genes of cytokines may influence the production level. These SNP have been studied extensively in the context of acute rejection after organ transplantation. Very different results have been obtained by different groups. Drawbacks of most studies are often the limited sample size, allowing analysis of just one cytokine at a time rather than of all cytokines of interest. In order to abolish this size problem, a concerted action of all centers performing these studies should be needed. Such a joint effort would enable analysis of SNP in all the cytokine genes simultaneously in a large cohort of organ donors and recipients. The main focus in most studies has been SNP genotype of the recipient. But it is becoming increasingly clear that, in addition o the recipient's immune system, the microenvironment of the donor organ also contributes to cytokine production, thus influencing rejection events. More attention should be paid to the role of this microenvironment and cytokine production by the cell types present. In our opinion, this is an essential way by which the role of the cytokine network and of cytokine nucleotide polymorphisms in acceptance and rejection of organ grafts should be further elucidated. The exact influence of immunosuppressive therapy on SNP and expression of the cytokines is so far not clear. Furthermore each organ has a different immunogenicity which might evoke a different immune response involving different cytokines. Efforts will have to be made to elucidate the complexity of the immune response towards different organs.

Table 1: Allele frequencies of SNP and repeat polymorphism in cytokine genes

Cytokine	SNP	Substitution		Allele	Frequency (%)
TNF-α	-308	G	A	G	75*
				A	25*
	-238	G	A	G	95*
				A	5*
IL-4	-590	C	T	C	73**
				T	27**
	-285	C	T	C	90***
				T	JO***
	-81	A	G	A	90***
				G	10***
IL-6	-174	G	C	G	63****
				C	37****
IL-10	-1082	G	A	G	21****
				A	49****
	-819	C	T	C	78****
				T	22****
	-592	C	A	C	78****
				A	22****
TGF-β	-800	G	A	G	92*****
				A	8*****
	-509	C	T	C	66*****
				T	34*****
	Codon10	T	C	T	60*****
				C	40*****
	codon25	G	C	G	94*****
				C	6*****

* Allele frequencies as determined by Fanning et al. [95]
** Allele frequencies as described by Rosenwasser et al.[52]
*** Allele frequencies as described by Song et al. [53]
**** Allele frequencies as described by Marshall et al. [40] / [54]
***** Allele frequencies as described by Cambien et al. [91]

Table 2: Allele frequencies of SNP in cytokine genes determined in a panel of 70 cardiac recipients and 61 cardiac donors

Cytokine	SNP	Allele	Frequency (%)	
			Recipient	Donor
TNF-α	-308	G	81	82
		A	19	18
	-238	G	98	94
		A	2	6
IL-4*	-590	C	84	89
		T	16	11
	-285	C	100	100
		T	0	0
	-81	A	100	100
		G	0	0
IL-10**	-1082	G	49	55
		A	51	45
	-819	C	82	78
		T	18	22
	-592	C	82	78
		A	18	22
TGF-β ***	-800	G	97	95
		A	3	5
	-509	C	56	58
		T	44	42
	“codon10	T	69	67
		C	31	33
	codon25	G	90	92
		C	10	8

* Bijlsma et al. [95]
** Bijlsma et al. [86]
*** Bijlsma et al. [95]

Table 3: Complete listing of relations described in literature for cytokine gene SNP and acute rejection after organ transplantation

Cytokine	SNP allele	Production		Relation acute rejection Heart/ Kidney/Liver	Reference
TNF-α	-308*A	High	Heart	+	[32, 35]
	-308*G	Low	Heart	-	[33]
	-308*G/A		Heart	None	[34]
	-308*G/A		Kidney	None	[40, 54]
IL-4	-590T	High	Heart	_	[86]*
	-590*C/T		Kidney	None	[40, 54]
	-590*T		Kidney	-	[55]
IL-6	-174*G/C		Heart	None	[33]
	-174*G/C		Heart	None	[35]
	-174*G/C		Kidney	None	[40]
	-174*G		Kidney	-	[54]
	-174*G/C		Kidney	None	[37]
IL-10	-1082*G	High	Heart	_	[11,33,73]
	-1082*G/A		Heart	None	[86]
	-1082* A	Low	Liver	+	[80]
	-1082*G	High	Kidney	+	[82, 83]
TGF-β	-800/-509		Heart	None	[95]**
	10/25		Heart	None	[95]**
	10/25		Heart	None	[33]
	10/25		Kidney	None	[37,40,54,55]
	10/25		Liver	None	[80, 85]

*and ** Bijlsma et al. [86, 95]

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3

TNFα in patients with end-stage heart failure on medical therapy or supported by a Left Ventricular Assist Device.

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Abstract

Background: In the heart elevated levels of TNFα can cause lethal heart failure, like Dilated Cardiomyopathy (DCM). The level of TNFα production is in part determined by promoter gene polymorphisms. We investigated whether the TNFα promoter gene polymorphism is in this way involved in the outcome of end-stage heart failure and predicts whether patients require left ventricular assist device (LVAD) support or can be kept on medical therapy (MT)while awaiting heart transplantation (HTx). As most patients in this study received a heart transplant, the role of the TNFα polymorphisms in transplant rejection was studied as well.

Methods and Results: In twenty nine patients with DCM, 35 patients with Ischemic Heart Disease (IHD; both on MT), 26 patients on LVAD support and 61 cardiac transplant donors TNFα plasma level was detected by EASIA. In both patients groups high levels of TNFα plasma levels was observed however, in patients supported by LVAD this increase was much higher compared to patients on MT. Furthermore, this increase seems to be associated with the TNF1 allele ('G' at position –308) instead of the TNF2 allele (A at position –308). The promoter polymorphisms at position –238, -244 and –308 were observed by polymerase chain reaction and sequencing. Polymorphism at positions –238, -244 and –308 did not show any relevant differences between the groups. Howver, at position –308, a trend of a higher incidence of the TNF2 allele (an "A" at position -308) in DCM patients compared to donors was shown. The distribution of the TNF1 and TNF2 alleles was not different in patients on medical therapy compared to the patients supported by a LVAD. No association was found between patients' TNFα promoter gene polymorphism and rejection. However, patients that received a donor heart with the TNF2 allele developed more rejection episodes, compared to patients that received a donor heart with the TNF1 allele.

Conclusion: TNFα levels are high in patients with end stage heart failure on MT, but even higher in patients on LVAD support. This high TNFα plasma level however, is not correlated with the TNF2 allele but seems to be associated with the TNF1 allele. Furthermore, in HTx the donor TNFα gene seem to play a more important role in severity of acute rejection than that of the patient.

Introduction

Cardiovascular disease is the most common cause of death in industrialised nations. Although the death rate from myocardial infarction has declined, the incidence of end-stage heart failure has increased [1]. Beside Ischemic heart disease (IHD), dilated cardiomyopathy (DCM) is a major cause of end-stage heart failure, which is characterised by ventricular dilatation and systolic contractile dysfunction. Heart transplantation (HTx) has proven to be an effective long-term treatment for these patients. However, due to the shortage of donor organs some patients with severe end-stage heart failure need to be supported with a left ventricular assist device (LVAD) as bridge to HTx [2-4].

Proinflammatory cytokines are up regulated in patients with heart failure and have been implicated in the pathophysiology of this disease [5, 6]. TNFα, a proinflammatory cytokine, is produced by monocytes and macrophages, but also by activated T-cells, fibroblasts, mast cells and NK cells in response to inflammatory or infectious stimuli [7]. Even cardiomyocytes in the failing human heart produce TNFα [8-10]. Low physiologic levels of TNFα confer a cytoprotective effect in the heart [11, 12], whereas high levels of TNFα modulate cardiovascular functions through a variety of mechanisms, such as inducing left ventricular dysfunction [13], left ventricular remodeling [14], abnormalities in myocardial metabolism [15], and cardiomyocyte apoptosis [16]. Transcription of TNFα is regulated by transcription factors that bind to transcription sites in the promoter region. Nucleotide variability (polymorphisms) in this promoter region may influence this transcription, and as a consequence the level of TNFα production. In the TNFα gene a lot of different polymorphisms and microsatelites are known, but at least three of them are located in the promoter region and do influence the production of TNFα. In all three polymorphic sites the common nucleotide "G" is substituted by an "A" [17-19]. Wilson et al. described that the "A" at position –308 (indicated as TNF2 allele) constitutes a much more powerful transcriptional site than the "G" at position –308 (indicated as TNF1 allele). Although, no nuclear protein that binds to the polymorphic site is known, they suggested that the polymorphism has a direct effect on the transcriptional activity. They demonstrated a six to sevenfold higher level of transcription from the TNF2 allele in in vitro experiments [20]. As different studies (7,9) have shown that TNFα plays an important role in the pathogenesis of end-stage heart failure, we hypothesized that the promoter polymorphism in the TNFα gene influences the development of end-stage heart failure. Because LVAD support can help the patients with the most severe heart problems that would otherwise die on medical therapy awaiting HTx, we investigated whether patients supported by a LVAD carry more often alleles associated with a high TNFα production, than patients on medical therapy. As all patients in our study population received heart transplantation, we also investigated the influence of the TNFα promoter gene polymorphisms on graft rejection.

Materials and methods

Patient population

The effect of TNFα polymorphisms on heart failure in 29 patients suffering from end stage DCM (mean age: 45 ± 12) and 35 patients with IHD (mean age: 51 ± 8) both medical therapy prior to heart transplantation (HTx), were compared with twenty-six patients (15 with IHD, mean age: 45 ± 9; 11 with DCM, mean age 33 ± 14) which were supported with a pneumatic or electric LVAD (Heart-mate, Thoratec, Pleasanton, California) as bridge to HTx. All patients received a heart transplantation (HTx) between 1999 and 2004.

Sixty one HTx donors were used as control group. All patients were on ACE inhibitor treatment in the stable phase of heart failure. At the time of LVAD implantation, all patients were in NYHA functional class IV, and in NYHA functional class I while on LVAD support. Before LVAD implantation the ACE inhibitor treatment was stopped and all patients received intravenous inotropics because of hemodynamic deterioration. Cardiac medication was discontinued initially in all patients after LVAD implantation. However, in 30% of patients at some stage hypertension was treated by ACE inhibitors again. All of the patients, after HTx were treated with Cyclosporine A, Azathioprine, and Prednisolone triple therapy [21]. Informed consent to participate in this study was obtained from all patients.

Rejection scores of the patients

Rejection episodes were diagnosed by histopathological examination of endomyocardial biopsies, according to the criteria of the International Society for Heart and Lung Transplantation [22]. HTx patients in this study with a grade 3A or higher were defined as rejectors. Patients with grade 0, 1 and 2 were defined as non-rejectors.

TNFα plasma concentrations

TNFα plasma levels were detected with EASIA (Biosource Europe S.A., Nivelles, Belgium) following the manufacturers' instructions. Plasma levels were measured pre-HTx or pre-LVAD.

DNA isolation of the patients and donors

DNA of the patients was obtained from peripheral blood lymphocytes (PBL). DNA of donors was isolated from mononuclear cells from spleen, isolated by Ficoll density gradient centrifugation. DNA was isolated according the salting-out procedure [23]

Identification of the TNFα gene polymorphism and genotyping

Polymorphisms at positions -238, -244 and -308 in the TNFα promoter region were identified by Polymerase Chain Reaction (PCR) and subsequent sequencing. PCR was performed using the upstream primer, 5'gaaacagaccacagacctg3' and the downstream primer,

5'ctcacactccccatcc3', that were both selected with the computer program Oligo (National Biosciences Inc., Plymouth, USA). The PCR mixture contained 500 ng DNA, 1x PCR buffer (Perkin Elmer, Roche Molecular Systems, Inc., Branchburg, New Jersey), 2 mM MgCl2 , 0.8 mM dNTPs (Promega Corp., Madison, Wisconsin), 10 pmol upstream primer, 10 pmol downstream primer and 0.75 U AmpliTaq DNA Polymerase (Perkin Elmer) and finally overloaded with mineral oil. Cycling was performed at 94°C for 3 min, followed by 35 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 2 min, with a final cycle of 72°C for 5 min in a ABI-thermocycler 480 (Perkin Elmer, Foster City, CA). PCR products were purified prior to sequencing to inactivate unincorporated PCR primers and deoxynucleotide triphosphates by enzymatic treatment. This was accomplished by mixing 6 μl exonuclease 1 (10 units/μl; Amersham, Buckinghamshire, UK) and 1 μl shrimp alkaline phosphatase (2 units/μl ; Amersham) and incubating at 37°C for 30 min followed by 80°C for 20 min. Sequencing was performed by using dye terminator cycle sequencing ready reaction kit (Perkin Elmer). Analysis was done by the computer program ABI PrismTM Sequencing 2.1.1.

Statistical analysis

All data were calculated using the paired Wilcoxon signed rank test or the Mann Whitney test of the statistical package of Prism 3.02 for Windows. A p-value < 0.05 was considered statistically significant.

Results

TNFα plasma levels in patients on MT and in patients on LVAD support.

TNFα plasma levels were measured in patients on MT (n = 64) and in patients on LVAD support (n = 26). Patients with end-stage HF either on MT or LVAD support showed significantly increased TNFα levels compared to the healthy controls (Figure 1; p = < 0.001). In patients supported with a LVAD this increase is much higher compared to the patients on MT (p = 0.05) and this increase was augmented in patients which expressed the GG genotype of the TNFα promoter polymorphism at position -308. No significant differences in TNFα plasma concentration was observed in patients expressing the GA genotype, either on MT or LVAD support compared to the control or between the 2 groups of patients.

TNFα promoter polymorphism in heart failure patients

As shown in table 1, 29 patients with DCM on medical therapy (MT), 35 patients with IHD on MT, 26 patients on LVAD support and 61 donors were screened for their TNFα promoter polymorphism at position –238, -244, -308.

The A polymorphism at position –238 was found in 1/29 (3 %) patients with DCM on MT, 2/35 (6 %) patients with IHD on MT and in 7/61 (12 %) of the donors. These differences

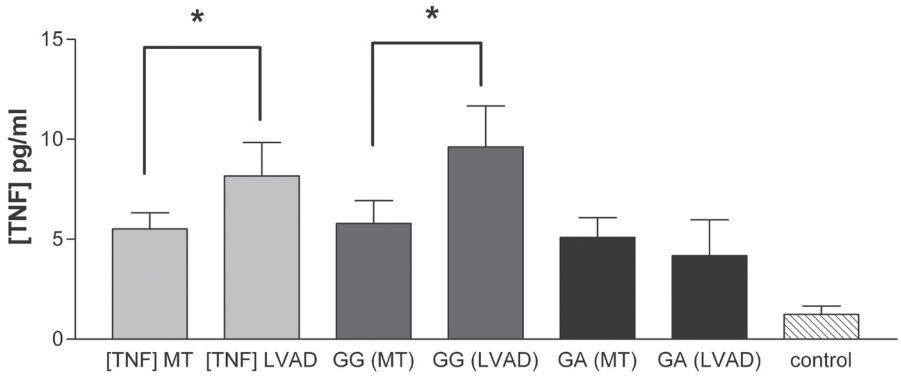

Figure 1. TNFα plasma levels in patients on medical therapy or LVAD support prior to heart transplantation and their promoter polymorphism on position -308. TNFα plasma levels were significantly increased both in patients on medical therapy (MT) and on LVAD support compared to healthy controls. This increase was even higher in patients with the G polymorphism. * $p < 0.05$.

between the patient and donor groups were not significant. In the patients on LVAD support the A polymorphism at position -238 was found in 2/11 (18%) of the patients with DCM and in 0/15 (0%) of the patients with IHD.

The A polymorphism at position –308 (TNF2) was found in 13/29 (45%) of patients with DCM on MT, in 10/35 (29%) of patients with IHD on MT and in 17/61 (28%) of donors. In patients with DCM a higher frequency of the TNF2 allele was observed compared to IHD and donors. This trend, however was not significant ($p = 0.15$). In patients on LVAD TNF2 was found in 4/11 (36%) of the patients with DCM and in 1/15 (7%) of the patients with IHD.

No significant differences were found in the TNFα polymorphism distribution between patients on MT and patients on LVAD support.

The A polymorphism at position –244 was not found in any of the patients or donors (data not shown). This substitution of a "G" to "A" at position –244 was observed almost exclusively in North American Blacks (23). Our patient and donor groups consisted only of Caucasians.

Table 1: TNFa promoter polymorphism at position –238 and –308 in patients and donors.

Heart failure	Position -238			Position -308		
	GG	GA	AA	GG	GA	AA
DCM (MT)	28 (97%)	1 (3%)	0	16 (55%)	12 (41%)	1 (3%)
IHD (MT)	33 (94%)	2 (6%)	0	26 (74%)	9 (26%)	1 (3%)
DCM (LVAD)	09 (82%)	2 (18%)	0	07 (64%)	04 (36%)	0
IHD (MT)	14 (93%)	1 (6%)	0	14 (93%)	01 (6%)	0
Donor	54 (89%)	7 (12%)	0	44 (72%)	15 (25%)	2 (3%)

The number of patients and donors are given. Within brackets: the frequency of each genotype at that position in patient and donor group is given. DCM = Dilated cardiomyopathy, IHD = Ischemic heart disease, MT = medical therapy, LVAD = left ventricular assist device.

Association of TNFα promoter polymorphism with heart transplant rejection

Most patients included in this study underwent a heart transplantation.

Only three patients and 7 donors have the A polymorphism at position –238. A correlation could therefore not statistically be defined. Therefore, we correlated the rejectors and non-rejectors during the first six months post heart transplantation with the polymorphism at position –308. There was no significant difference between the TNF1 and TNF2 allele in the rejectors and non-rejectors. However, as shown in fig. 2, patients who received a donor heart with the TNF2 allele, where more often defined as rejectors, compared to those which received a donor heart with the TNF1 allele (p = 0.03).

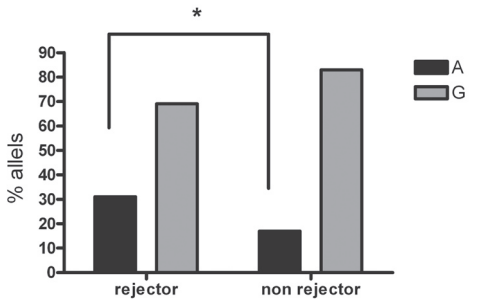

Figure 2. Frequency of the TNFα promoter polymorphism at position –308 of the donors in relation to rejection. Patients receiving the A polymorphism had more often rejection episodes than patients receiving the G polymorphism. * p = 0.03. Rejector ≥ grade 3, non rejectors < grade 3

Discussion

In this study, we showed that patients with end-stage HF on LVAD support do express significant higher levels of TNFα compared to patients on MT. However, this increased TNFα production is not correlated with the TNF2 polymorphism, but seems to be associated with the TNF1 polymorphism. Furthermore, we showed that patients that received a donor heart with the TNF2 polymorphism had more severe rejection episodes during the first six months after HTx then patients that received a donor heart with TNF1 polymorpism

TNFα is produced in the heart by many cells including cardiomyocytes [8-10]. It has been shown that TNFα is up-regulated in the myocardium in response to various forms of cardiac injury, and in addition, plays an important role in the physiology of end-stage heart failure [5]. The use of LVAD in end-stage heart failure has increased over the last 2 decades, since these devices became generally available [24]. In the majority of cases LVAD have been used as a bridge to transplantation. However, in some cases successful explantation of the LVAD was reported [25-29]. Unloading of the heart by a LVAD lead to reversal of LV dilatation, to regression of LV myocyte hypertrophy and to neurohormonal changes [4, 30-32]. Patients supported with a LVAD have generally the most severe heart failure and would not survive on medical therapy awaiting HTx. Our hypothesis was that since TNFα plays an important role in the development of end-stage heart failure, that the patients supported by LVAD produce more TNFα and therefore carry more often the TNF2 allele compared to patients that were on medical therapy. In line with our hypothesis, measurement of the TNFα plasma concentration in patients with end-stage heart failure showed that patients who required

LVAD support expressed more TNFα protein in their plasma compared to the patients on medical therapy. However, this over expression of TNFα is not linked to TNF2, but seems to be associated with TNF1 expression. In the present study 29 patients with DCM and 35 patients with IHD which were on medical treatment while waiting for HTx, were screened for their promoter polymorphism and compared to 26 patients with severe end stage heart failure supported with a LVAD. The TNF2 was found in 45% of the patients with DCM and 29% of the patients with IHD. This indicated a over expression of the TNF2 allele in patients suffering for DCM, but this trend was not significant. This result suggest that TNFα plays a more important role in DCM then IHD. Densem et al (28) also studied the TNFα promotor polymorphism in the relation to end-stage heart failure. They did find a significant overrepresentation of the TNF2 allele in the patients with DCM compared to patients with IHD and controls. These data support our data, as in our relative small groups of patients a similar trend was observed. Interestingly, patients suffering from DCM on LVAD support expressed more often the "A" polymorphism at position -238 compared to patients with DCM on MT (18% compared to 3%). The influence of the polymorphism at the position -238 is not clear yet, but it is possible that this polymorphism can influence the TNFα production as well. Wilson et al showed a six to sevenfold higher level of transcription from TNF2 in in vitro experiments. It may suggest that the serum levels of TNFα do not correlate with the intra myocardial concentrations of TNFα. TNFα is a proinflammatory cytokine which is released by macrophages and T-cells at the site of inflammation, causing endothelial cell activation, up-regulation of cell adhesion molecules, increasing vasodilatation and local vascular permeability, which will lead to TNFα release in the blood stream. Nevertheless, serum concentrations may not reflect TNFα production in the heart, as other source could be involved as well. TNFα could also play a role in the severity of rejection after HTx, although conflicting data consist about the role of TNFα and allograft rejection have been described [33-35]. Several studies have suggested that the TNFα polymorphism at position -308 modulate the rejection risk [36, 37]. We did not detect a significant difference between the polymorphism in the rejectors and non-rejectors. However, we did show that patients that received a donor heart with TNF2 had more severe rejection episodes during the first six months after HTx than patients that received a donor heart with TNF1. Also the group of Hutchinson et al has described that the genotype of the donor is important in chronic rejection [38]. Most studies that did report an association between the rejection episodes and the TNFα polymorphism reported a polygenic risk assessment with an IL10 polymorphism [36, 37, 39] . However, Bedi et al [40] did not detect any association with the TNF2 polymorphism and an increased risk of allograft rejection. Comparing the various studies on HTx and TNFα polymorphisms it has to be realized that there is a large variation in approach. 1) the complications are not consistent. 2) Some groups define acute rejection as ISHLT grading ≥ 2, while others as ≥ 3A. 3) Non rejectors were defined as no acute rejection while others included patients with grading 1 and 2. 4) Time in which the rejection episodes occur was different between studies, and 5) Differences in immunosuppressive regimes.[41]

Taken all data together this study showed that TNFα is an important factor in the development of heart-failure and in line with our hypotheis in patients who required LVAD support, higher TNFα plasma levels were detected compared to patients on MT and healthy controls. However, this TNFα production is not associated with the TNF2 allele, but seems to correlate with the TNF1 allele. Furthermore, the TNFα polymorphism of the donor seems to play an important role in tranplant rejection. Unfortenuatly, our data on TNFα gene polymorphismhowever do not reach significance. A large international multi center study is necessary to increase the patient group to see whether the trend become significant. Furthermore, TNFα is a complicated cytokine which acts not on its own but is part of a multiplex of factors including other cytokines and growth factors.

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4

Reverse remodeling of the myocardial extracellular matrix after prolonged Left Ventricular Assist Device support follows a biphasic pattern

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Abstract

Background: Collagens are important components of the extracellular matrix (ECM). Alterations in collagen structure and composition can lead to end-stage heart failure. Left Ventricular Assist Devices (LVAD) are frequently used as bridge to heart transplantation (HTx). In this study, we analyzed changes in composition of the collagens as well as the synthesis or degradation of these collagens after prolonged LVAD support.

Methods and results: The ECM volume was quantified after Picro-Sirius red staining. With immunohistochemistry (IHC) type I and III collagen protein and with quantitative-PCR collagen mRNA expression was analyzed. Collagen synthesis and degradation was studied by measuring N-terminal propeptide for type I collagen (PINP), N-terminal propeptide for type III collagen (PIIINP) and carboxyterminal telopeptide for type I collagen (ICTP) in plasma and the collagen composition with the hydroxyproline/Sircol assay. The ECM volume increased in the first 200 days after LVAD implantation. Between 200 and 400 days the ECM volume decreased, but remained higher than pre-LVAD. After 400 days the ECM volume was smaller than the pre-LVAD volume. IHC did not show a significant difference pre- and post-LVAD in collagen composition. Collagen mRNA expression did not change but an augmented synthesis of collagen during the first month after LVAD support was measured by the plasma PINP and PIIINP levels. Besides, the quality of the collagen network improved.

Conclusion: Reverse remodeling during LVAD support follows a biphasic pattern. Initially, an increase of type I and type III collagen turn over takes place, which is paralleled by a volume increase of the ECM. Subsequently, this turnover reduces and the ECM volume decreases, together with a restoration of the collagen network.

Introduction

The myocardial extracellular matrix (ECM) consists of a fibrillar collagen network, in which the total amount, location, and architecture of the collagens are of functional importance. Discontinuities within this network, or its disruption, will result in the loss of its structural support function, and ultimately lead to a set of changes in myocardial geometry and function, designated as structural remodeling[1]. In patients with end stage heart failure, support of the left ventricle with a left ventricular assist device (LVAD) results in neurohormonal changes [2-4], improvement of the patients general condition, reduction in cardiac size (reverse remodeling), and a limited recovery of the contractile elements [5-9]. The structure of the ECM after LVAD support has been the subject of several studies. In most of these, an increase in ECM volume was observed [10-14], whereas in others a decrease was found [15, 16]. In studies reporting an increased ECM volume, the patients had been on LVAD support for a relative short period of time. Of the many components which constitute the ECM, collagen fibrils and proteoglycans are the most abundant ones [1, 17]. Fibrillar type I collagen represents 85%, and type III collagen 11% of the total collagens in the cardiac ECM [18, 19]. Type I and type III collagens are secreted as pro-collagens. From these, the amino terminal propeptides (PINP, PIIINP) are cleaved off, prior to incorporation of the new collagen molecules into collagen fibers. When collagen is degraded by matrix metalloproteinases (MMP), the carboxyterminal telopeptide is cleaved off (ICTP). Cardiac collagen turnover in cardiac tissue repair and fibrosis can be determined 'at distance' by measurement of biological markers such as PINP, PIIINP and ICTP as has been shown both in experimental models [20] and in clinical conditions [21-26]. The aim of the present study was to analyze the changes in the ECM with particular emphasis on type I and type III collagen, after short (0-200 days) and prolonged LVAD support (200-400 days and onwards). The studied materials were heart biopsies taken before and after LVAD support. Our study provides evidence indicating that the reverse remodeling in the ECM follows a biphasic pattern.

Material and Methods

Patients and materials

Subjects of this study were 26 patients (age: 37 ±12 years; 21 men and 5 women) with refractory end-stage HF (Patients characteristics are summarized in table 1). They were all treated with a pneumatic or electric LVAD (Heart-mate, Thoratec, Pleasanton, California) as a bridge to heart transplantation (HTx), between March 1993 and May 2002. Twenty-four of these patients were successfully transplanted. Two patients died before HTx, one as a result of recurrent cerebral embolism and the other due to chronic ischemic enteritis. At the time of LVAD implantation, all patients were in NYHA functional class IV, and in NYHA functional

class I while on LVAD support. All patients were on ACE inhibitor treatment in the stable phase of heart failure. Before LVAD implantation these were stopped and all patients received intravenous inotropics because of hemodynamic deterioration. Cardiac medication was discontinued initially in all patients after LVAD implantation. However, in 30% of patients at some stage hypertension was treated by ACE inhibitors again as indicated in table 1. Informed consent to participate in this study was obtained from all patients. The myocardial biopsy at the time of LVAD implantation consisted of the LV apical core, which was removed during LVAD implantation. These biopsies (pre-LVAD) were compared with LV tissue specimens of the explanted heart after HTx (post-LVAD), taken from the apical half of the LV, outside the suture area of the inflow canula. In the two patients that died, tissue specimens of the heart at autopsy were studied. All biopsies were directly fixed in 4% buffered formalin and embedded in paraffin. Furthermore, in 14 cases, tissue was directly frozen after LVAD implantation and after HTx. Plasma samples of 11 patients were collected and frozen at –135°C until use. The plasma samples were taken before LVAD implantation, and 1, 3, and 6 months after LVAD implantation and at time of HTx. Plasma samples of 7 healthy volunteers were used as control.

Table 1: Patients' Characteristics

Patient	Gender	Age	Disease	Days on LVAD support	Medication during LVAD support
1	Female	30	DCM	66	Enalapril
2	Female	39	DCM	69	Enalapril
3	Male	30	DCM	77	None
4	Male	23	DCM	102	Amlodipine
5	Female	27	DCM	124	Enalapril
6	Male	19	DCM	178	Amlodipine, Enalapril
7	Female	36	DCM	178	None
8	Female	52	DCM	187	None
9	Male	53	DCM	190	None
10	Male	53	DCM	242	Amlodipine, enalapril
11	Male	23	DCM	297	None
12	Male	21	DCM	348	None
13	Male	25	DCM	399	Captopril
14	Male	39	DCM	433	None
15	Male	18	DCM	455	Amlodipine
16	Male	49	IHD	35	None
17	Male	53	IHD	71	Amlodipine
18	Male	42	IHD	72	None
19	Male	28	IHD	97	None
20	Male	48	IHD	106	None
21	Male	52	IHD	113	Amlodipine
22	Male	34	IHD	137	Amlodipine
23	Male	40	IHD	254	None
24	Male	32	IHD	300	None
25	Male	54	IHD	347	Enalapril
26	Male	38	IHD	557	Enalapril

Cross-sectional area of the cardiomyocytes.

The cardiomyocyte cross-sectional area was measured as described previously by De Jonge et al [7]. In short, the cross-sectional area of at least 50 cardiomyocytes per each sample was measured on 3 µm slides stained with modified azan, making use of a Videoplan morphometric program (Zeiss Kontron, Eching, Germany)

Measurement of ECM volume

The ECM volume was measured morphometrically on 3 μm paraffin embedded slides stained with Picro-Sirius red, using the Optimas 5.2 software system (Dutch Vision System, Breda, The Netherlands). With the staining technique, cardiac myocytes stained yellow and components of the ECM stained red. ECM volume was expressed as the percentage of all areas stained red within the slide; blood vessels were excluded. Large, sharply fibrotic areas (scar tissue) were identified and taken to be caused by infarction. Care was taken to analyze only those areas that were remote from scar tissue

Collagen composition of ECM

The composition of the ECM and the localization of type I and III collagens in heart tissue sections was performed on paraffin embedded heart sections, using a standard two-step immunohistochemical analysis [3]. Pepsin was used as antigen retrieval system. Primary monoclonal antibodies were anti- type I collagen and anti- type III collagen (both diluted 1:60; Neomarkers, Fremont, USA). Subsequently, the slides were incubated with mouse powervision (Klinipath, Duiven, the Netherlands) as a second antibody.

Quantitative PCR for type I and III collagens

In order to determine the expression of collagen mRNA in heart tissue, RNA was isolated from frozen tissue sections (20x 10 μm) using TRIzolTM Reagent (GibcoBRL, Rockville, USA). Three μg of RNA was taken for cDNA synthesis using oligo-dT and random primers. Five μl of a 1:30 dilution of the cDNA was used in the Assay-on-Demand kit (Applied Biosystems, CA, USA) for quantitative PCR (Taqman P7700, Applied Biosystems). Total volume was 25 μl. The following thermo cycle profile was used for type I and type III collagen and PBGD (house keeping gene): 95°C for 10 min followed by 45 cycles of 95°C for 15 sec and 60°C for 1 min. Each sample was run in duplicate. The following formula was used to quantify the amount of mRNA in our samples: relative quantity = $2^{-\Delta\Delta Ct}$, $\Delta\Delta Ct = \Delta Ct$ (sample) - ΔCt (calibrator) and ΔCt = Ct (target) – Ct (reference). The calibrator sample consisted of cDNA of placenta, and was measured in parallel and in all experiments. In this way it served as an inter-run comparison which makes it possible to compare the data of the various experiments. [4] This is indicated as relative abundance.

PINP, PIIINP and ICTP detection with a Radio-Immuno-Assay (RIA)

To determine collagen synthesis and breakdown after LVAD implantation, plasma levels of

the aminoterminal propeptides PINP and PIIINP and the carboxyterminal telopeptide ICTP of 11 patients pre-LVAD and 1, 3, and 6 months during LVAD support and at HTx were determined by a Radio-Immuno-Assay according the manufacturer's instructions (RIA; Orion Diagnostica, Finland).

Sircol collagen assay / Hydroxyproline assay

Frozen myocardial tissue was fractionated into pepsin-soluble and -insoluble collagens. The myocardial pepsin-soluble collagens were extracted overnight with 5 mg/ml pepsin in 0.5 mol/L acetic acid [27]. The soluble and insoluble collagens were separated by centrifugation at 2,100 g for 6 min at 4°C. The soluble collagens can be further separated into undenatured and denatured collagens, after which the undenatured collagen can be quantified with a Sircol collagen assay kit (Sanvertech, Heerhugowaard, The Netherlands). The denatured, total soluble, and insoluble collagens were hydrolyzed and measured by determining the hydroxyproline content (Hypronosticon kit; Organon Teknika, The Netherlands). The data are expressed as μg collagen per mg wet tissue weight, assuming that interstitial collagens contain an average of 12,7% hydroxyproline [28].

Statistical analysis

The data from the Picro-Sirius red staining, PCR, RIA, and the Hydroxyproline/Sircol tests were analyzed using the paired Wilcoxon signed rank test, Mann Whitney-U tests or One-way ANOVA (Bonferroni's Multiple Comparison Test), when appropriate. All data were calculated with the statistical package of Prism 3.02 for Windows. A p-value < 0.05 was considered statistically significant.

Results

Because biopsies pre-LVAD (apex) and post-LVAD (LV wall) were taken from different anatomical locations, we examined biopsies of the complete LV circumference of 4 patients. The type I and III collagen contents of the various regions within the explanted heart did not differ significantly. Furthermore, none of the data analyzed in this study showed any significant differences between patients with DCM and patients with IHD. Therefore, both patient groups are taken as a single one. Data are presented as mean ± SD.
Data on patient characteristics, LVAD support duration and medication are summarized in Table 1.

Decrease of the cross sectional area of the cardiomyocytes

The average cardiomyocyte cross sectional area decreased significantly by 33% during LVAD support. Only 3 out 26 patients showed an increase in cardiomyocyt size. As shown in Figure 1A the maximum reduction was already present after 100 days of LVAD support (pre-LVAD 884 ± 487 μm^2; post-LVAD 576,4 ± 316 μm^2, $p<0.0001$)

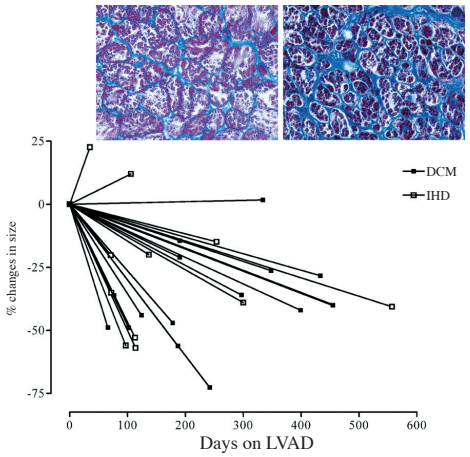

Figure 1A Change in cross-sectional area of the cardiomyocyte after LVAD support. The size at LVAD implantation was set at zero. Percentage size change was calculated at time of HTX (days on LVAD)(n = 26). Inserts: representative Modified Azan staining in heart tissue sections from a patient after 124 days on LVAD: (I) pre-LVAD and (II) post-LVAD. Note the decrease of cardiomyocyte size and increase of collagen content (cardiomyocyte stained red and collagen stained blue). No significant difference was observed between IHD (open squares) and DCM (filled squares) patient group.

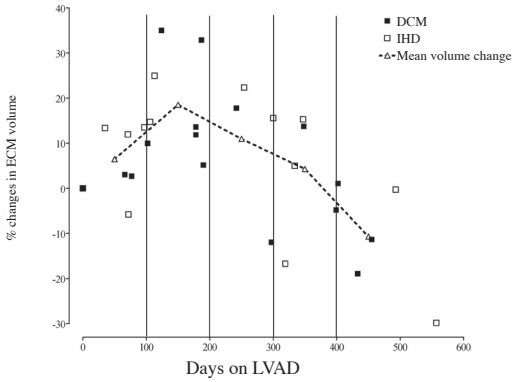

Figure 1B. Change in extracellular volume after LVAD support. The ECM volume at implantation was set at zero. Percentage change in ECM volume was calculated at time of HTX (days on LVAD) (n = 26). ECM volume changes are divided into 100 days increments. Δ- Δ: the mean of the ECM volume changes after 0-100, 100-200, 200-300, 300-400 and >400 days on LVAD. No significant difference was observed between IHD and DCM patient groups (n = 26).

Biphasic changes in the ECM volume

The volume of the ECM in the biopsies, as measured using Picro-Sirius red, increased significantly in the whole group (pre-LVAD 23.4 ± 16.7% versus post-LVAD 32.3 ± 16.9%; $p < 0.05$) after LVAD support. To analyze the changes in ECM volume, the period after LVAD implantation was divided arbitrary in periods of 100 days. As indicated in figure 1B, in almost all patients the ECM volume increased between 0 and 200 days. Between 200 and 300 days and 300 through 400 days, the volume increase tapered off but the ECM volume was still larger than pre-LVAD. After 400 days, however, the ECM volume was smaller than pre-LVAD. The period after which this ECM reduction starts is probably dependent on a variety of factors and therefore varies between individual patients.

Changes in ECM composition of type I and type III collagen

All myocardial biopsies showed a prominent IHC staining for type I and type III collagen in the perimysium (surrounding groups of cardiomyocytes). Type III collagen appeared more abundantly than type I collagen (data not shown). Similar semi-quantitative differences between type I and type III collagen existed in the endomysium (surrounding each individual cardiomyocyte), but they were less prominent than in the perimysium. In contrast, in infarcted areas type I collagen was most abundant (data not shown). No obvious differences were observed between the proportions of type I and type III collagen, pre- and post-LVAD support.

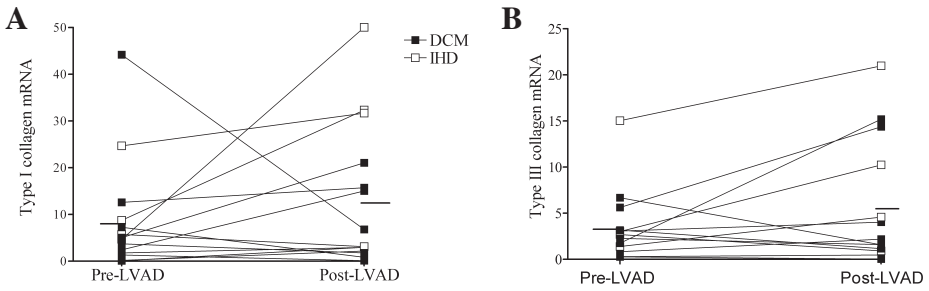

Figure 2. Type I and type III collagen mRNA expression pre-LVAD support and at heart transplantation.
Relative abundance of type I collagen mRNA (A; n = 14) and type III collagen mRNA (B; n = 14). There was no significant increase of mRNA content after LVAD support (p = 0.17). However, in 9/14 patients an elevated mRNA expression was observed. No significant difference was observed between IHD and DCM patient group. Horizontal bars are the mean values.

Quantitative PCR for Type I and III collagen mRNA

The quantities of type I and type III collagen mRNA were compared in RNA from frozen biopsies taken pre- and post-LVAD. In most patients (9/14), the relative abundance of mRNA increased for both type I and III collagen after LVAD support (Figure 2). However, this increase was not significant when the group was taken as a whole (p = 0.17). There was no correlation between the relative abundance of type I and III collagen mRNA and the duration of LVAD support (data not shown).

PINP, PIIINP and ICTP levels in plasma

For type I and type III collagen synthesis, the aminoterminal propeptide PINP and PIIINP was measured in the plasma of patients pre-LVAD and 1, 3 and 6 months post-LVAD and at time of HTX. The carboxyterminal telopeptide ICTP was measured in plasma at the same time points, as a measure of type I collagen degradation (Figure 3). Plasma concentrations of PINP and PIIINP were augmented significantly in the first months after LVAD implantation, and subsequently decreased gradually during LVAD support; they never returned to the plasma levels of healthy persons (Figure 3, p < 0.01). Plasma ICTP was high before LVAD support compared with the healthy controls, and decreased during LVAD support but never reached control levels (p <0.001).

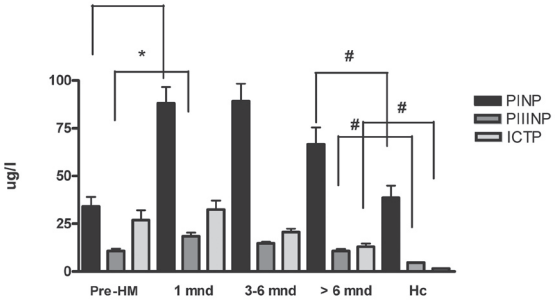

Figure 3. Type I and III collagen serum markers during LVAD support (n=7). Hc = healthy control. * p<0.01, # p<0.001.

Sircol collagen assay / Hydroxyproline assay

The hydroxyproline assay was performed to measure the insoluble and soluble collagen fractions in pre- and post-LVAD samples. The undenatured soluble collagen fraction was determined using the Sircol assay. After LVAD support, no significant change was found in

Ratio	Pre-LVAD	Post-LAVD	HC
Insoluble/ total soluble	0.07 ± 0.04	0.06 ± 0.01	0.09 ± 0.06
Total soluble/total collagen	0.93 ± 0.03	0.94 ± 0.01	0.92 ± 0.05
Undenaturated/total soluble	0.18 ± 0.06	0.36 ± 0.10$^{\#}$	nd

total collagen and total soluble collagen (data not shown). The undenatured collagen content of the soluble fraction was increased after LVAD support; as shown in Table 2, the ratio of undenaturated to total soluble collagen was doubled after LVAD support. This is indicative for improvement of the collagen structure.

Discussion

In this study, it is shown that changes in the ECM volume in patients with cardiac failure after LVAD support follow a biphasic pattern. The ECM volume increases in the first period (0-200 days) after LVAD support; during prolonged support, this volume gradually decreases again (from 200-400 days and onwards). In many patients, type I and type III collagen mRNA expression increased after LVAD support, in parallel with a significant increase in type I and III collagen synthesis (PINP, PIIINP). This resulted in a regeneration of the collagen network after LVAD support, as indicated by an improved collagen quality.

Conflicting data exist on the changes in ECM volume. Some have observed an increase of the ECM after LVAD support, some a decrease, and some others did not observe any change in collagen content after LVAD support. [10-14, 16, 29, 30]. These discrepancies may be explained by differences in treatment regimens, and duration of LVAD support. Furthermore, collagen and ECM detection methods differed between the studies. To the best of our knowledge, this is the first study that correlates various changes in the ECM in patients supported by LVAD, for both a short and long period of time. First, this study showed that the cardiomyocyte size decreased in almost all patients (23/26) in the first 100 days almost to normal [7]. After 100 days, no further reduction observed. This is in contrast to the changes in ECM volume. In the initial period (0-200 days) after LVAD support, the ECM volume increased. In patients supported for more than 200 days, this increase in ECM tapered off and

even decreased again after support duration of more than 400 days. Our data on the ECM volume fit with literature data [10-14]. In these studies, LVAD support was given for a relative short period of time (up to 200 days). Madigan et al [12] described a time-dependent increase in ECM volume during the first 40-80 days, after which the volume stabilized. When LVAD support was given for a longer period of time (18-420 days) a decreased ECM volume was observed by Bruckner et al [16]. Milting et al [30] found either an increase or a decrease in collagen amount as measured by 4-Hydroxyproline content, but in this study no correlation with time was made. We did not find a change in total collagen content, as measured by the hydroxyproline and Sircol methods. However, the apparent quality of collagen did improve over time, since the undenatured soluble collagen and the ratio of undenatured to total soluble collagen was significantly increased after LVAD support. Similar data have been described by others [29, 31]. The total ECM volume appeared to increase in the initial phase after LVAD support, but the total collagen content did not. This is strengthened by the fact that we did not observe a significant difference in collagen protein content (by IHC) after LVAD support. Nevertheless, there is an active turn-over of the collagens as indicated by the serum PINP and PIIINP levels and a slightly but non significant increase in degradation of type I collagen as measured by ICTP in the serum in the first 100 days of LVAD support. This collagen turnover tapered off after longer periods of LVAD support, but were still significantly elevated at time of HTX compared to healthy controls. During the initial period after LVAD support, the changes in serum collagen markers may certainly be influenced, to an unknown extent by the total body remodeling which undoubtly takes place after LVAD implantation. Consequently these data should be interpreted with care. Nevertheless, the serum collagen markers at a later stage correlated closely with the changes in the heart, as was also described by others in heart failure conditions [21, 32-34]. Since the initial increase of ECM volume is not paralleled by increased collagen content, it is likely that this Sirius red staining positive ECM consists of a loose type of connective tissue of poorly cross-linked collagens and other ECM components such as proteoglycans. The subsequent reduction in ECM volume after longer periods of LVAD support (> 400 days), may be due to the improved quality of the collagen network. This coincides with a size reduction of the heart, after ventricular unloading [31, 35]. From the total body of data the following picture emerges. Initially LVAD support and unloading of the heart induces a strong reduction of cardiomyocyte size. This size reduction of the cardiomyocytes has to be compensated for in the ventricular wall, which results in an increase in ECM volume, which does not necessarily imply the occurrence of fibrosis. After unloading and adaptation to this new condition a slow process of remodeling is started (with individual variation in course over time), which will finally result in a reduction of the ECM and a reduction in heart size. Cardiac medication was discontinued in all patients at LVAD implantation. However,during LVAD therapy, in 30% of patients at any stage high blood pressure was treated by ACE inhibitors again. As ACE inhibitors may affect collagen turnover [36, 37], we investigated whether the differences found in our study could

be attributed to this treatment. No significant differences in ECM volume, however, were observed between patients receiving ACE inhibitors after LVAD implantation and patients without ACE inhibitors. Therefore, we have no indication that our findings can be attributed to the discontinuation of the ACE inhibitors in the majority of our patients. The impact of the unloading by the LVAD on the ECM is therefore more important than the use or discontinuation of ACE inhibitors. No differences were observed between patients with DCM or IHD. In most studies regarding ECM changes after LVAD support both DCM and IHD are included, but differences between these two patients groups have not been mentioned [10, 30, 38]. Madigan et al [12] concluded that the ECM changes were independent of the cause of congestive heart failure. However, McCarthy et al [13] described an increased fibrosis selectively in DCM patients. Li et al [29] described differences in collagen content (mainly in patients with DCM), as detected by the hydroxyproline assay. Also on this level no significant differences were detected in our patient groups.

In conclusion, our results indicate that in the first period of LVAD support, the size of the cardiomyocytes is reduced, and an active turnover of the collagens in the ECM takes place. Despite the observed increase of the ECM volume after 0-200 days of LVAD support, there is no increase in total collagen content. Therefore, the synthesis and degradation of collagens are in equilibrium, and the increase of the ECM volume is not so much due to collagen, but probably due to other components of the ECM (such as proteoglycans), which are stained by Picro-Sirius red as well. Furthermore, after prolonged LVAD support, the ECM volume is reduced, which is paralleled by a significant regenerative improvement of the collagen network. This indicates that despite the severely advanced heart failure, the heart is still capable of a regenerative response, which holds promise for future regenerative therapies.

Study limitations:

None of the serum markers we used is heart specific. Our study population was free of known diseases that affect collagen turnover. One could hypothesize, however, that because the patients' conditions improved markedly after LVAD implantation, this might influence the collagen metabolism at other sites in the body than in the heart alone. We cannot rule out that the changes in the collagen serum markers as seen in the first month could be related to the improvement of performance and total body remodeling as a consequence of the LVAD implantation. However the changes seen in the months thereafter are at least in part indicative for ECM remodeling in the heart because we have histological proof of remodeling in the heart during this period.

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Type IV collagen degradation in the myocardial basement membrane after unloading of the failing heart by a Left Ventricular Assist Device

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Abstract

Background: After Left Ventricular Assist Device (LVAD) support in patients with end stage cardiomyopathy, cardiomyocytes decrease in size. We hypothesized that during this process, known as reverse remodeling, the basement membrane (BM), which is closely connected to, and forms the interface between the cardiomyocytes and the extracellular matrix, will be severely affected. Therefore, the changes in the myocardial BM in patients with end-stage heart failure before and after LVAD support were studied. Also the role of MMP-2 in this process was investigated.

Methods and results: Transmission Electron Microscopy showed that the BM thickness decreased post-LVAD compared to pre-LVAD. Immunohistochemistry indicated a reduced immunoreactivity for type IV collagen in the BM after LVAD support. Quantitative PCR showed a similar mRNA expression for type IV collagen pre- and post-LVAD. MMP-2 mRNA almost doubled post-LVAD ($p<0.01$). In addition, active MMP-2 protein as identified by gelatin zymography and confirmed by Western blot analysis was detected after LVAD support and in controls, but not before LVAD support. Active MMP was localized in the BM of the cardiomyocyte, as detected by type IV collagen in situ zymography. Furthermore, in situ hybridization / immunohistochemical double staining showed that MMP-2 mRNA was expressed in cardiomyocytes, macrophages, T-cells and endothelial cells.

Conclusion: Taken together, these findings showed reduced type IV collagen content in the BM of cardiomyocytes after LVAD support. This reduction is at least in part the result of increased MMP-2 activity and not due to reduced synthesis of type IV collagen.

Introduction

Left ventricular assist devices (LVAD) are commonly used in patients with heart failure as a bridge to heart transplantation (HTx). In most cases, LVAD support extends the patient's life span and improves the quality of life [1, 2]. In addition, pressure and volume unloading of the left ventricle (LV) by LVAD can reverse left ventricular dilatation and leads to regression of left ventricular hypertrophy and neurohormonal changes [3-6]. The understanding of this process, referred to as 'reverse remodeling' [7] is important for a better insight in both myocardial events during LVAD support and the processes leading to heart failure. Besides, several institutions described the possibility of LVAD explantation without the need for heart transplantation (weaning)[4, 8-11]. This requires a profound knowledge of the process of reverse remodeling.

In previous studies with cardiac unloading, we demonstrated partial recovery of the contractile myofilaments in the cardiomyocytes [12] and decreased natriuretic peptide levels both in the plasma of the patients, as well as in the heart [5, 6]. Since reverse remodeling not only involves the cardiomyocytes but also the extracellular matrix (ECM), we have focused on the changes in ECM before and after LVAD support. The ECM, which consists of the fibrillar collagens type I and type III collagen and comprises a basement membrane (BM) surrounding cardiomyocytes, forms a continuum between different cell types within the myocardium and provides a structural supporting network to maintain myocardial geometry [13, 14]. We have shown that reverse remodeling of type I and type III collagen in the ECM follows a biphasic pattern [15]. Initially, an increase of type I and type III collagen turn over took place, which was paralleled by a volume increase of the ECM. Subsequently, this turnover reduced and the ECM volume decreased, together with a restoration of the collagen network. Type IV collagen, which is one of the components of the BM [14] differs from the interstitial collagens by the presence of globular domains interspersed within the triple-helical segments [16]. This unique feature gives type IV collagen the flexibility to assemble into a sheet like network. Therefore, type IV collagen in the BM links the sarcolemma of individual myocytes to the surrounding ECM and interstitial cells [17] and is important in the transmission of force during diastole and systole. The function of laminin, another component of the BM, includes the mediation of adhesion, migration, growth and differentiation of cells [18-20].

Degradation of the basement membrane proteins occurs under a variety of physiological and pathological circumstances including embryogenesis [21], wound healing [22] and metastasis [23] by matrix metalloproteinases (MMPs). It is known that MMP-2 is able to degrade type IV collagen and is up-regulated during heart failure [14, 24, 25]. Because we have shown changes in cardiomyocyte size and ECM volume after unloading of the LV by LVAD support [12, 15] and given the close connection between type IV collagen and the cardiomyocyte, we hypothesized that the BM surrounding the cardiomyocyte must be re-shaped when the cardiomyocytes become smaller by unloading of the heart by a LVAD. If so, MMP-2

(collagenase IV) can play a major role in this process of remodeling. Therefore, in heart biopsies taken before and after LVAD support, the BM was studied with transmission electron microscopy (TEM), type IV collagen mRNA expression by quantitative PCR (Q-PCR), and the presence of type IV collagen protein by immunohistochemistry (IHC). MMP-2 was studied by Q-PCR, in situ hybridization (ISH), IHC, Western- blotting (WB), gel- and in situ zymography (localization and identification of active enzymes).

Material and methods

Patients

In this study 25 patients with refractory end-stage heart failure were included. Characteristics of these patients are summarized in table 1. All patients were treated with a LVAD (Heart-mate, Thoratec, Pleasanton, California) as a bridge to transplantation. Twenty-three of these patients were successfully transplanted. Two patients died before HTx; one as a result of recurrent cerebral embolism, the other due to chronic ischemic enteritis. All patients were in NYHA class IV at the time of LVAD implantation, and in NYHA class I while on LVAD support. Informed consent to participate in this study was obtained from all patients. The myocardial biopsy at time of LVAD implantation consisted of the LV apical core removed during implantation. These biopsies (pre-LVAD) were compared with LV tissue specimens of the explanted hearts after HTx (post-LVAD) from the apical half of the LV, outside the suture area of the inflow cannula. In patients with IHD care was taken to use myocardium away from the infarct zone, the 'unaffected' myocardium. In the two patients who died before transplantation corresponding tissue specimens of the heart at autopsy were used. All biopsies were directly fixed in buffered formalin and embedded in paraffin. In addition, in 11 cases (pre- and post-LVAD) biopsies were frozen in liquid nitrogen after implanting the LVAD or after HTx, and in 10 cases (pre- and post-LVAD) biopsies were fixed in Karnovsky's fixative for transmission electron microscopy (TEM) analysis. Control tissue was taken from the left ventricle of non-used donor hearts (n=6). From 3 of these hearts also samples were available for TEM evaluation.

Transmission electron microscopy (TEM)

Heart tissue and biopsies of the LV were fixed in Karnovsky fixative, followed by 4% OsO4. After dehydration in alcohol they were embedded in epon, following routine procedures.
Material was selected (unaware of the patient's background details) for longitudinal fiber localization in semi-thin sections, stained with toluidine blue and viewed by a light microscope. Ultrathin sections were stained with 5% uranyl acetate and 2.5% lead citrate, randomly analyzed and photographically recorded under a TEM (Jeol 1200 EX-2). Measurement of the thickness of the BM was performed with Image J 1.25 (NIH, USA). Of all 10 patients and 3

Table 1. Patients characteristics

Patient	Gender	Age	Disease	Days on LVAD support	Medication during LVAD support
1	Female	30	DCM	66	Enalapril
2	Male	30	DCM	77	None
3	Male	23	DCM	102	Amlodipine
4	Female	27	DCM	124	Enalapril
5	Female	36	DCM	178	None
6	Male	19	DCM	178	Amlodipine, Enalapril
7	Female	52	DCM	187	None
8	Male	53	DCM	190	None
9	Male	53	DCM	242	Amlodipine, Enalapril
10	Male	23	DCM	297	None
11	Male	21	DCM	348	None
12	Male	25	DCM	399	Captopril
13	Male	39	DCM	433	None
14	Male	18	DCM	455	Amlodipine
15	Male	49	IHD	35	None
16	Male	53	IHD	71	Amlodipine
17	Male	42	IHD	72	None
18	Male	28	IHD	97	None
19	Male	48	IHD	106	None
20	Male	52	IHD	113	Amlodipine
21	Male	34	IHD	137	Amlodipine
22	Male	40	IHD	254	None
23	Male	32	IHD	300	None
24	Male	54	IHD	347	Enalapril
25	Male	38	IHD	557	Enalapril

control hearts the BM of 10 cardiomyocytes was measured. Of each cardiomyocyte the median of 12 measurements was taken and the mean of all 10 cardiomyocytes from one patient/control was calculated.

TEM photographs (at least 17 for each patient) showing both collagen fibers and sarcolemma with a BM were analyzed. The number of photographs showing collagen but no contact with the BM and the photographs showing single contacts with the BM or showing bundles connected to the BM were counted and expressed as the % of all pictures.

For all analyses the observer was blinded to the study group (pre or post-LVAD or control) she studied.

MMP macro array and Quantitative PCR for different MMP

To characterize the gene expression profile of 23 different MMP in one RNA sample, RNA was isolated from frozen tissue sections using TRIzolTM Reagent (GibcoBRL, Rockville, USA). Isolated RNA was directly used in the MMP GeArray kit (SuperArray, Frederick, MD,

USA) following the GeArray User Manual. Differences in MMP and TIMP expression before and after LVAD support were calculated using the software included in the GeArray kit.
To confirm the MMP mRNA expression on the MMP GeArray, RNA was isolated from frozen myocardial tissue sections using TRIzolTM Reagent for Q-PCR. Three μg of RNA was used for cDNA synthesis using oligo-dT and random primers. Five μl of a 1:30 dilution of the cDNA was used in the Assay-on-Demand kit for MMP-1, -2, -8, -9, -13, -14, -15, -20, -25, -26 and TIMP-1, -2, –3 and -4 (Applied Biosystems, Foster city,CA, USA) for Q-PCR (Taqman P7700, Applied Biosystems). Porphobilinogen deaminase (PBGD) and 18S were chosen as references. Total volume was 25 μl. The following thermo cycle profile was used for the MMP, PBGD and 18S (house keeping genes): 95°C for 10 min followed by 45 cycles of 95°C for 15 sec and 60°C for 1 min. Each sample was run in duplicate. The following formula was used to quantify the amount of mRNA in our samples: relative quantity = $2^{-\Delta\Delta Ct}$,, $\Delta\Delta Ct = \Delta Ct$ (sample) - ΔCt (calibrator) and $\Delta Ct = Ct$ (target) – Ct (reference). The calibrator sample was cDNA from human placenta tissue, which was run with each experiment and therefore, it was possible to make an inter-run comparison.

Quantitative PCR for type IV collagen α1-α6

To determine the mRNA expression of the different alpha chains of type IV collagen in heart tissue, RNA was isolated from frozen tissue sections using TRIzolTM Reagent (GibcoBRL, Rockville, USA). Three μg of RNA was used for cDNA synthesis using oligo-dT and random primers. Five μl of a 1:30 dilution of the cDNA was used in the Assay-on-Demand kits for all six alpha chains of type IV collagen (Applied Biosystems, CA, USA) in a Q-PCR assay (Taqman P7700, Applied Biosystems). Porphobilinogen deaminase (PBGD) was used as reference gene.

MMP-2 RNA in situ hybridization (ISH) and ISH / IHC double staining

To identify cell types expressing MMP-2 mRNA, RNA ISH and RNA ISH / IHC double staining was performed as described by van Hoffen et al [26]. MMP-2 and IL2 were amplified using specific primers (MMP-2 forward: 5'ATTCCGCTTCCAGGGCACATC 3' and MMP-2 reverse: 5' GTTAAAGGCGGCACCACTCG 3'). The PCR products were labeled with digoxigenin as described previously. The specificity of the probes for ISH was confirmed with tonsil as positive control. Specificity of the ISH for MMP-2 was further controlled by using a probe of similar composition and labeling directed against Interleukin-2 (IL2) (26). Negative controls were obtained by overnight RNAse pre-treatment of sections of heart tissue at 37°C (RNAse A 100 µg/ml, RNAse T1 100 U/ml, Boehringer –Mannheim GmbH), and by omission of the probe from the hybridization mixture. For ISH/IHC double staining procedure was performed as described by Bruggink et al [5], using antibodies against CD3 (T-cells), CD68 (macrophages) and Factor 8 (endothelium) and the MMP-2 probe

Immunohistochemistry of type IV collagen, laminin and MMP-2

To localize type IV collagen, laminin and MMP-2 protein expression, a two-step immunohistochemical analysis was performed [12, 27] on paraffin embedded sections. Pepsin was used as antigen retrieval. Primary antibodies used were: type IV collagen (polyclonal; 1:100 Novocastra laboratories Ltd, Newcastle upon Tyne, UK), laminin (polyclonal; 1: 200) and MMP-2 (monoclonal; 1:20; both Neomarkers, Fremont, USA). For type IV collagen a second polyclonal antibody [28] was used as second opinion. Subsequently, the slides were incubated with rabbit powervision (Klinipath, Duiven, The Netherlands). The specificity of the IHC was confirmed by positive and negative controls. Placenta was used as positive control. Omission of the primary antibody and replacement of the primary antibody by isotype-matched irrelevant antibodies served as negative controls.

Western blotting MMP-2

Immunoblotting for MMP-2 was performed on proteins isolated from myocardial biopsies before and after LVAD support. Equal amounts of protein were separated on a 12% Bis-Tris polyacrylamide gel (Bio-Rad Laboratories, Veenendaal, The Netherlands) and transferred to nitrocellulose membrane. Western blot analysis was performed with a rabbit polyclonal anti-MMP-2 (Santa Cruz Biotechnology, California, USA) antibody (1:500) in TTBS containing 1% BSA. After incubation with horseradish peroxidase-conjugated secondary antibody, the blot was developed using Amersham ECL system (Amersham Pharmacia Biotech Europe GmbH). Alpha-actin was used as loading control. The intensity of the bands were quantified with the computer program TotallabV 2003.03 (Nonlinear Dynamics, Newcastle upon Tyne, UK)

Gelatin Zymography

Cardiac tissue was homogenized in 1x sample buffer containing 0.5% Triton X-100 (Sigma, Zwijndrecht, The Netherlands), 0.5 U/ml aprotinin (Sigma) and 0.01% sodium azide in PBS. The samples were centrifuged and the supernatants were collected and stored at –20°C until use. MMP enzyme expression was assayed by SDS-PAGE zymography using gelatin as substrate. Equal volumes of samples were subjected to electrophoresis, without boiling or reduction, through an 8% polyacrylamide gel co-polymerized with gelatin (2 mg/ml). After electrophoresis was completed the gels were washed twice in 2.5% Triton X-100 rinsed in aquadest and incubated overnight in Brij 35 solution (50mM TRIS-HCl pH 7.4, 10 mM CACl2 and 0.05% Brij-35). Finally, the gels were stained using Coomassie Blue (Sigma). Proteolytic bands were visualized by de-staining the gels with 25% MeOH and 15% acetic acid in MiliQ and quantified with the computer program TotallabV 2003.03.

Type IV collagen in situ zymography

To demonstrate active MMP-2 in frozen heart tissue sections, ISZ was performed according to Rouet-Benzineb et al [29]. Lysis of the substrate at the side of active MMP resulted in

fluorescence, assessed by fluorescent microscopy or confocal laser scan microscopy. To test whether fluorescence was specific, some slides were pre-incubated with a MMP inhibitor (phenanthroline monohydrate (20 μg/ml; Sigma) in Tris-HCl) and incubated with the substrate in combination with the inhibitor. All slides were examined by two investigators in a random blinded manner. The fluorescence was graded, as: 0 = negative, 1 = weakly positive, 2 = positive, 3 = strongly positive, 4 = very strongly positive. Values for each slide were obtained by taking the mean of all scored areas.

Statistical analysis

Grading of the ISZ, Q-PCR data, and BM measurements were compared using the paired Wilcoxon signed rank test or the Mann Whitney test. Inter- and intra- observer variability was tested using kappa analysis. All data were calculated with the statistical package of Prism 3.02. A p value < 0.05 was considered significant.

Results

Since staining for type IV collagen, laminin and MMP showed no significant differences between patients with DCM and patients with IHD (excluding the infarcted areas), both patient groups are considered as one.

Given the difference in localization of the pre-LVAD myocardial biopsies (apex) and the post-LVAD biopsies (apical half of the LV), we examined whether regional differences were present in the heart by analyzing longitudinal sections (7 biopsies of LV, 4 of RV and 4 of septum) of several hearts. Using IHC (4 hearts) and ISZ (2 hearts) we did not observe grading differences between the various cardiac areas. The inter- and intra observer kappa values for ISZ were 0.58 and 0.60, respectively, and for TEM 0.77 and 0.77, indicating fair agreement.

Transmission electron microscopy.

LV biopsies of the apex (pre-LVAD) and LV wall (post-LVAD) were used for TEM (n = 10 patients). In biopsies taken pre-LVAD support the BM of the cardiomyocytes was irregular in size and in most cases dispersed; the lamina lucida and lamina densa were difficult to discriminate (1A, 1C). In biopsies taken after LVAD support a clear lamina lucida and lamina densa of the BM was observed (figure 1B, 1D). Two types of connections of the collagen fibers to the BM were discriminated: contacts of single collagen fibers or bundles of collagen fibers. In the biopsies taken before LVAD support both single contacts as well as bundles of collagen fibers connecting the BM were observed (figure 1A,1C,and 1G). In contrast to the pre-LVAD biopsies, the connections of collagen bundles to the BM and the total number of contacts were significantly decreased post-LVAD ($P < 0.05$; figure1B, 1D and 1G). In normal hearts the BM showed the same irregular structure as the pre-LVAD BM. Also the collagen-

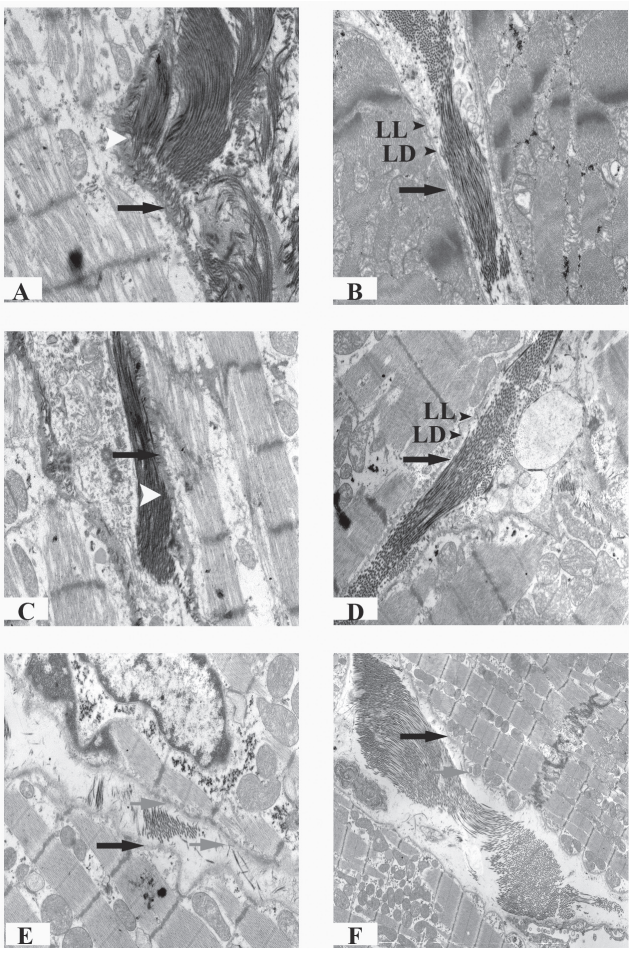
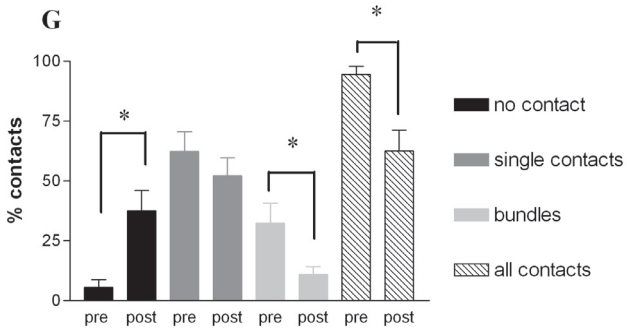


Figure 1. Basement membrane structure and thickness as observed with transmission electron micrsocopy before and after LVAD support. Representative examples (n = 10) of TEM of heart biopsies. Figure shows 2 patients with end-stage heart failure, pre-LVAD support: A and C; post LVAD: B and D. A (12 K) and C (10 K): Pre LVAD, notice the irregular size of the BM and vague contours of the lamina lucida (LL) and lamina densa (LD) but also the tight connections of the ECM with the BM pre-LVAD. B and D: Post-LVAD (both 10K) the LL and LD could be clearly distinguished but there was also a lack of connection of the ECM with the BM. E (10 K) and F (4 K): control (n = 3). Notice the irregular size of the BM and the clear contacts between the BM and the collagens fibers. * $p < 0.05$. Black arrow: BM; White arrow heats: bundles of collagen fibers connecting to the BM. Grey arrows: single collagen fibers connecting to the BM.



G: Quantification of different contacts between collagen fibers in the ECM and the basement membrane. Indicated is the % of photographs showing collagen but no contact with the BM (no contact) and the photographs showing single contacts with the BM (single contacts) or showing bundles connected to the BM (bundles).

BM contacts are similar between normal hearts and pre-LVAD (Figure 1E and F). The thickness of the BM decreased from 0.09 ± 0.02 μm pre-LVAD to 0.07 ± 0.02 μm ($p = 0.04$) after LVAD support. The thickness of the BM in the healthy control group was 0.08 ± 0.0006 μm (figure 1G).

MMP macro array and Quantitative PCR

The MMP macro array data showed that multiple MMP and TIMP were expressed in heart tissue (n = 11, figure 2A,B) pre- and post-LVAD support. Moreover, some MMP displayed an altered expression after LVAD support: MMP-2 was significantly increased ($p = 0.04$) after LVAD support, however MMP-24 decreased significantly ($p < 0.01$) after LVAD support.
Most of the array data were confirmed by Q-PCR. MMP-2 significantly increased both in the macro array and by Q-PCR ($p < 0.01$, Figure 2C). However, MMP-24 did not change after LVAD support as shown with Q-PCR ($p=0.3$). Furthermore, some MMPs which were present in the macro array, were almost not detectable in Q-PCR (MMP-13, MMP-20, MMP-26). The reason for the discrepancies was not clear. We considered the Q-PCR data more specific, as most MMP detected by the array were just above the detection limit and some cross reactivity could not be excluded of the arrays.

Type IV collagen α1-α6 mRNA expression in the heart.

Q-PCR for type IV collagen α1- α6 was performed using mRNA as templates from frozen heart biopsies, from patients with end stage heart failure pre and post-LVAD (n=11) and biopsies from control hearts (n=6; table 2). For all α1 to α6 chains of type IV collagen mRNA was present in the heart. There was no significant difference between expression levels of the six α-chains before and after LVAD support. However, although not statistically significant mRNA of the major collagen α3 and α4 chains tended to increase after LVAD support. Compared to the control group, post-LVAD the mRNA expression of the α3 chain was 2 times higher ($p = 0.02$), the expression of the α4 chain was significantly decreased, both in the pre- and post-LVAD support groups and the expression of the minor α6 chain was also decreased pre-LVAD compared to the control group ($p < 0.01$; table 2).

Table 2. Quantitative PCR for all the different α- chains of type IV collagen and MMP-2.
Type IV collagen and laminin protein expression before and after LVAD support

mRNA	Pre-LVAD	Post-LVAD	Control
Type IV collagen			
α1	0.6 ± 0.4	0.6 ± 0.6	1.2 ± 0.6
α2	0.2 ± 0.2	0.2 ± 0.2	0.2 ± 0.1
α3	13.0 ± 9.0	21.0 ± 12.0$^{\#}$	9.4 ± 8.8
α4	39.0 ± 36.0$^{\#}$	52.0 ± 37.0$^{\#}$	99.1 ± 34.4
α5	0.6 ± 0.5	1.0 ± 0.7	0.8 ± 0.3
α6	1.7 ± 1.5$^{\#}$	2.4 ± 1.7	2.7 ± 0.5
MMP-2	1.3 ± 0.7$^{\#}$	2.5 ± 0.8$^{*\#}$	0.4 ± 0.3

Data are presented as the mean value ± SD (relative abundance).

Relative abundance of mRNA as determined by Q-PCR of the different α-chains of type IV collagen and of MMP-2 in snap frozen myocardial biopsies from patients (n=11) with end-stage heart failure taken before (pre-) LVAD and at time of heart transplantation (post-LVAD), compared with biopsies taken from unused donor hearts (control; n=6). * $p < 0.01$ pre-LVAD vs. post-LVAD; # $p < 0.01$ pre-LVAD and or post-LAVD vs. control.

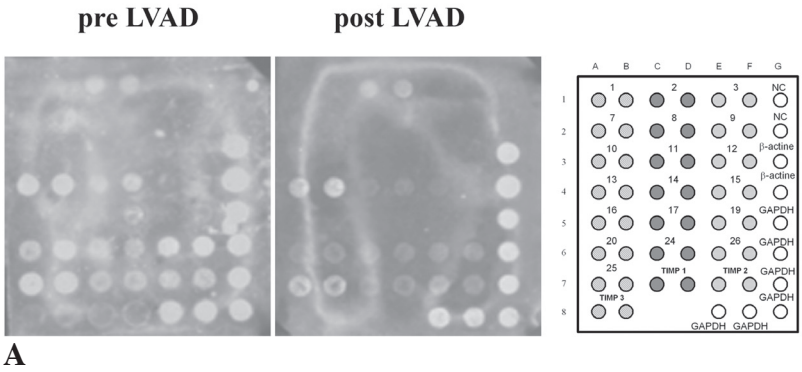


Figure 2. Macro-array and Q-PCR data for MMP and TIMP expression in pre and post LVAD heart tissue.
A. Representative example of pre and post LVAD macro-array analysis. Right panel shows the distribution of the various MMPs and TIMPs on the macro-array. Numbers correspond with the respective MMP. Also control spots are indicated. B. Quantification of macro-array data on expression of MMP and TIMP pre and post-LVAD (n=11). * p = 0.04 pre vs. post LVAD, # P < 0.01 pre vs. post LVAD. C. Q-PCR data of the expression of MMP and TIMP pre- and post-LVAD (n=11) All indicated MMP and TIMP were tested, so no bar present indicates no expression detected. * p < 0.01 pre vs. post-LVAD.

Formalin fixed biopsies from 25 patients before and after LVAD support were immunohistochemically stained for type IV collagen and laminin, both principal components of the BM.

Type IV collagen, Immunohistochemistry

Using two different antibodies, in all biopsies taken before LVAD support (figure 3A) type IV collagen was abundantly present in the basement membrane surrounding the cardiomyocyte and underneath the endothelial layer of blood vessels. All the biopsies taken after LVAD support showed a strong reduction or complete disappearance of staining of the BM surrounding the cardiomyocytes, for both type IV collagen antibodies tested, while the BM of the blood vessels remained positive for type IV collagen (figure 3B).

Laminin

Immunoreactivity of laminin was strongly present in the BM surrounding the cardiomyocytes in all biopsies taken before and after LVAD support. After LVAD support this immunoreactivity did not change significantly (figure 3C,D).

MMP-2 mRNA and protein expression in the heart during LVAD support

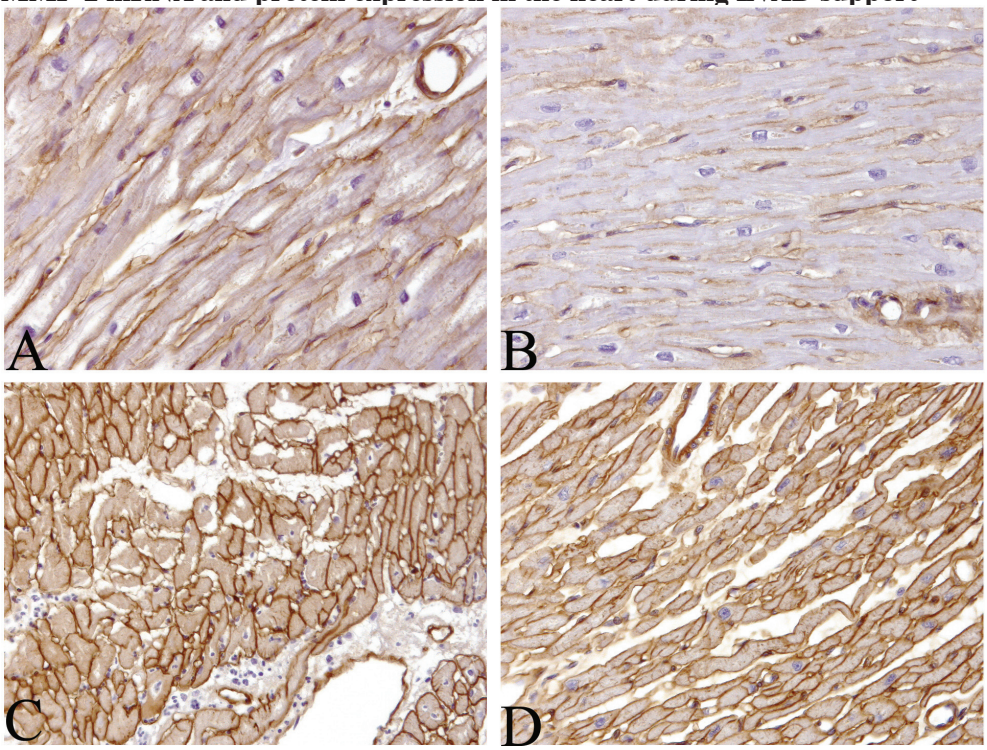


Figure 3. Type IV collagen and laminin immuunhistochemical staining of patients with end-stage heart failure pre- and post-LVAD support. A: Immunohistochemical staining of type IV collagen pre-LVAD (200x). B: A clear and consistent decrease of type IV collagen immunoreactivity was seen after post-LVAD (200x). C: Immunohistochemical staining of laminin pre-LVAD (200x). D: Post LVAD no differences in staining pattern of laminin was detected compared to pre-LVAD (200x).

Quantitative PCR

MMP-2 mRNA expression was almost doubled post-LVAD compared to pre-LVAD ($p = 0.01$, $n = 11$) as shown by Q-PCR. Furthermore, the MMP-2 mRNA expression showed 3 and 6 times higher levels compared to the control group ($p<0.01$ and $P<0.001$ respectively), in pre- and post-LVAD, respectively (table 2).

In situ hybridization

With MMP-2 mRNA in situ hybridization ($n = 11$) a weak staining in the sarcoplasma of the cardiomyocytes was observed. In the ECM the infiltrating cells and the endothelial cells in the blood vessels showed a positive staining for MMP-2 mRNA (figure 4A), the other ECM components were negative.

To show MMP specificity an IL-2 probe was used. As shown in figure 4B, this probe stained

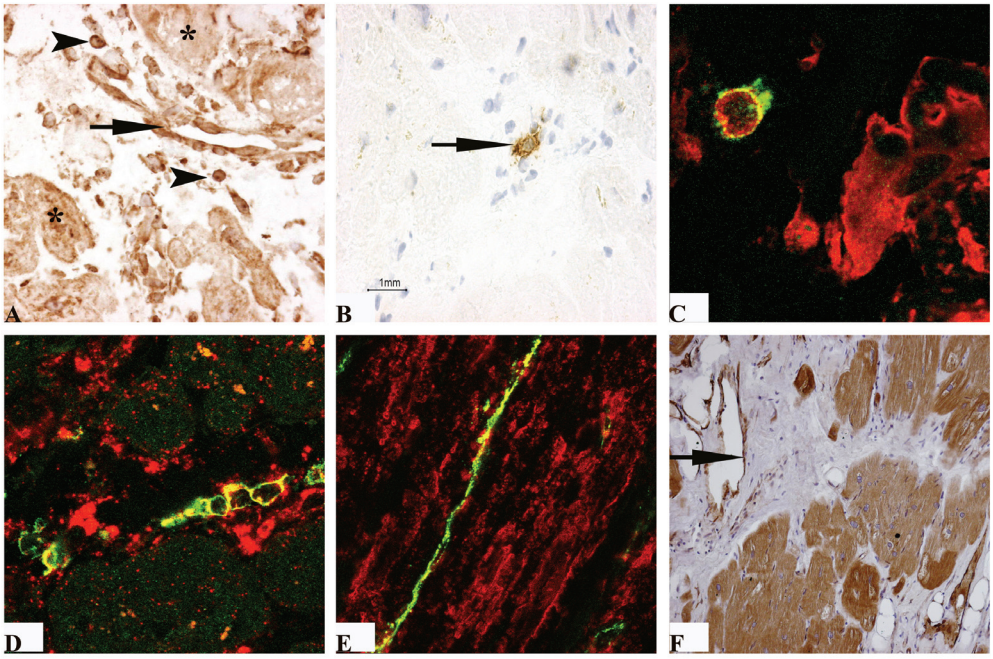


Figure 4. MMP-2 mRNA expression sites by in situ hybridization in the myocardium of patients with end-stage heart failure.
Representative examples ($n = 11$) of MMP-2 mRNA expression in myocardial biopsies taken before LVAD support. No significant change in expression was observed after LVAD support (not shown). A. Overview of MMP-2 in situ hybridization (200x). MMP-2 mRNA expression by endothelial cells of the blood vessel (arrow), infiltrating cells (arrow head) and the cardiomyocytes (astrix). B. Overview of IL2 in situ hybridization (200x). IL2 expression by infiltrating cells in the heart (arrow). No staining was observed in the cardiomyocytes and endothelium. C. Overlay of MMP-2 ISH-CD68 double staining (200x). MMP-2 in red and CD68 in green. CD68 positive macrophages expressed MMP-2 mRNA as was shown by the yellow color indicating MMP-2 and CD68 double staining. D. Overlay of MMP-2 ISH-CD3 double staining (50x). MMP-2 in red and CD3 in green. CD3-positive T lymphocytes expressed MMP-2 mRNA as was shown by the yellow color indicating MMP-2 and CD3 double staining. E. Overlay of MMP-2 ISH-factor 8 double staining (50x). MMP-2 in red and factor 8 in green. Factor 8 positive endothelium expressed MMP-2 mRNA as was shown by the yellow color indicating MMP-2 and factor 8 double staining. F. MMP-2 protein expression, as indicated by immunoreactivity in representative myocardial biopsy taken before LVAD support (200x, $n = 25$). Comparable results were obtained by ISH-IHC (double) staining as shown in B-D. No significant change in staining pattern intensity was observed after LVAD implantation (data not shown). Arrow = endothelial cells of a blood vessel.

a few lymphocytes present in the heart and not cardiomyocytes and endothelium as the MMP-2 probe did. Omitting the MMP-2 probe in the procedure, or RNAse treatment of the slides resulted in absence of positive staining.
In situ hybridization / IHC double staining was performed using monoclonal antibodies for CD3 (T-cells), CD68 (macrophages) and Factor 8 (endothelium), in combination with a MMP-2 labeled probe.
MMP-2/CD68: There was a considerable amount of macrophages present in the myocardium and almost all of these macrophages showed mRNA expression for MMP-2 (figure 4C).
MMP-2/CD3: In the myocardium only few T-cells were present both pre- and post-LVAD, and about half of these T-cells were positive for MMP-2 mRNA (figure 4D).
MMP-2/Factor 8: MMP-2 mRNA was abundantly present in Factor 8 positive endothelial cells in the myocardial capillaries and blood vessels (figure 4E). With both techniques (in situ hybridization and in situ hybridization/IHC double staining) no significant differences in MMP-2 expression and localization were observed between LV biopsies before and after LVAD support (data not shown)

Immunohistochemistry
Cardiomyocytes pre- and post- LVAD support expressed sarcoplasmic staining for MMP-2 by IHC (n = 25, figure 4F). The cardiomyocytes located in the trabecula and near the epicardium and endocardium stained stronger than the cardiomyocytes localized in the center of the biopsy. In the ECM, some fibrotic areas, fibroblasts, capillaries and infiltrating cells stained positive. In some cases the endothelial cells in the blood vessels and the coronary arteries were positive.

Increased MMP-2 activity after LVAD support
To quantify the MMP-2 protein expression in the heart, proteins isolated from heart tissue of 11 patients pre- and post-LVAD and 4 healthy controls were analyzed by Western blotting. Total MMP-2 did not change pre- vs. post-LVAD. However, the active form of MMP-2 (represented by the 62 kDa band) was significantly lower pre-LVAD compared to post-LVAD and the healthy control ($p = 0.04$ and $p = 0.01$ respectively; figure 5A). Post-LVAD the MMP- 2 levels returned to normal. The in-active form (as represented by the 72 kDa band) did not change significantly.
Gelatin-zymography was performed on extracts from frozen heart biopsies (n= 11). One band was detected in all pre-LVAD samples and two bands were detected post-LVAD (figure 5B). In the pre-LVAD sample the 72 kDa band was generated by inactive MMP-2. Post-LVAD, beside the inactive band an additional band, generated by active MMP-2 (62 kDa) was present. Quantification of the 72 kDa and 62 kDa bands showed a significant increase of both the inactive and the active form of MMP-2 post-LVAD ($p < 0.001$) compared to pre-LVAD. In normal heart tissue active MMP was also present as shown by the 62 kDa band. However, active MMP-2 was significantly higher post-LVAD than in the healthy control ($p <0.01$).
Active MMP is localized in the basement membrane.

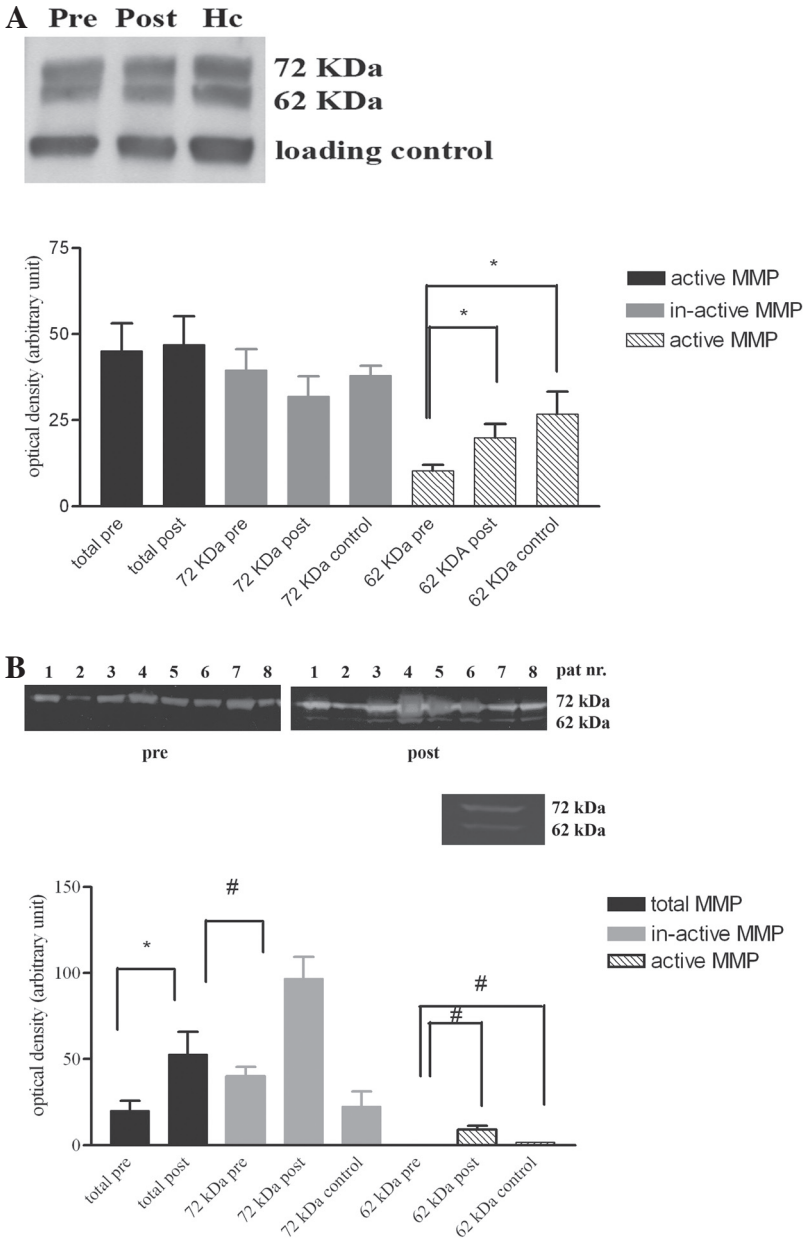


Figure 5. MMP-2 activity pre- and post-LVAD by western blot and gelatin zymography.
A: A representative immunoblot (n = 11) stained with anti-MMP-2 in myocardial biopsies pre-and post-LVAD support and controls (n = 6). The 72 kDa band represents inactive MMP-2 and the 62 kDa band represents active MMP-2. Quantification of the immunoblot, as presented in the bar graph, showed significant increase of active MMP-2 after LVAD support. (* $p = 0.04$).
B: Example of gelatin zymography gels showing MMP zymographic activity in 8 of 10 Pre-LVAD and post-LVAD samples. Inactive MMP-2 is represented by the 72 kDa band. Remarkably the active MMP-2 (represented by the 62 kDa band) was only present in the post-LVAD and control samples. The bar graphs shows quantification of the zymographic activity. There was a significant increase in total MMP and in both the inactive and active form of MMP-2 after LVAD support as compared to pre-LVAD and the healthy controls (* $p < 0.0001$, # $p < 0.01$).

Active MMP was localized using type IV collagen in situ zymography in frozen tissue sections from 11 patient's pre- and post- LVAD implantation. Type IV collagen ISZ showed in both pre- and post-LVAD samples a collagenolytic activity, localized in or near the basement membrane surrounding the cardiomyocyte and in the sarcoplasm of the cardiomyocyte (figure 6). The ECM showed no or only weak MMP activity. Semi quantitative grading of collagenolytic activity using a 0-4 grading scale did not show significant differences in membrane bound (2.5 ± 0.8 vs. 2.3 ± 0.5, ns) and sarcoplasmic (1.8 ± 0.6 and 2.0 ± 0.4, ns) activity in pre- vs. post-LVAD sample, respectively. However, ISZ is not a quantitative method, and mainly shows the localization of active MMP. After incubation of the tissue sections with a MMP inhibitor, no fluorescent signal was observed (data not shown), indicating that the collagenolytic signal was MMP specific.

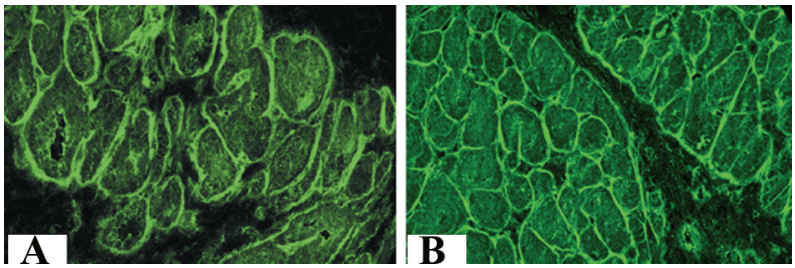


Figure 6. Type IV collagen In Situ zymography pre- and post-LVAD support
Representative examples (n = 11) of in situ zymographys of frozen biopsies pre- (A) and post-LVAD (B) (400x). Collagenolytic activity was prominently present in or near the BM of cardiomyocytes and to a lesser extend in the sarcoplasm. The ECM showed mostly weak activity. The pattern of staining was not different pre- and post-LVAD. Signal specificity was confirmed by pre-incubation with a MMP inhibitor (data not shown).

Discussion

Left ventricular force production is dependent on the coupling of myocytes to the extracellular matrix, which is mediated through the basement membrane. We hypothesized that in patients with end stage heart failure reverse remodeling during unloading of the left ventricle by a LVAD, consisting amongst others of reversal of cardiomyocyte hypertrophy, would be associated with remodeling of the basement membrane. The present study indeed demonstrates after LVAD support structural alterations in the basement membrane surrounding the cardiomyocytes, which was illustrated best by the decreased immunoreactivity for type IV collagen. Type IV collagen mRNA did not alter during LVAD support indicating that the decreased collagen was not due to a decreased synthesis. However, we detected increased MMP-2 mRNA and MMP-2 protein levels and MMP-2 activity as assessed by the presence of a 62 kDa band of active MMP in gel zymography and Western blotting after LVAD support suggesting an increased degradation of type IV collagen. Type IV collagen in situ zymography

indicated that the active MMP was located within the BM of the cardiomyocyte.
The novel finding in this respect is that after LVAD support most parameters return to a more "normal"situation. However, the basement membrane clearly shows an exception to this rule.

The use of LVAD's in end-stage heart failure has increased over the last 2 decades, since these devices became generally available [30]. The majority of the LVAD's have been used as a bridge to transplantation; however, in some cases successful explantation of the LVAD was reported [4, 8-11]. Unloading of the heart by a LVAD leads to reversal of LV dilatation and to regression of LV myocyte hypertrophy [1, 3, 12, 31]. The mechanisms of this reverse remodeling process are still largely unknown and therefore, the analysis of changes in the heart pre- and post-LVAD is important. The ECM, which consists of fibrillar collagens (Type I and Type III collagens) and a BM, forms a continuum between different cell types within the myocardium and provides a structural supporting network to maintain myocardial geometry. Several groups have studied the changes of the fibrillar collagens in the ECM in heart failure [32-34] and ECM after LVAD support. [35-37]. In a previous study of our group [15], we have shown that reverse remodeling of type I and III collagen in the ECM follows a biphasic pattern. Initially, an increase of type I and type III collagen turnover takes place, which is paralleled by an increase of the ECM volume. Subsequently, after a longer period of LVAD therapy this turnover reduces and the ECM volume decreases, together with a restoration of the collagen network. We hypothesized that the BM and therefore, type IV collagen and laminin must be remodeled during LVAD support because of the decreased cardiomyocyte size after LVAD support. This decrease in size was not biphasic, but reached its endpoint within the first 100 days on LVAD support (15). Indeed, LVAD support changed the morphology of the BM as assessed by TEM. The pre- LVAD biopsies from hearts with end-stage heart failure showed a thickened and irregular BM along the cardiomyocyte, and there were close connections between the collagen fibers in the ECM and the BM. Normal heart tissue showed a similar morphology. Post-LVAD the BM became more regular and the lamina lucida and lamina densa were compacter and could be clearly discerned. However, a disturbed connection between the BM and the collagen fibers in the ECM was observed. Although the exact consequences of these structural alterations are not known, it seems that connections between the collagen fibers and BM, benefit or are more easily formed, when the BM has a more irregular en thick structure. Interestingly, in contrast to most parameters showing normalization after LVAD support, the BM in normal hearts compares better with the pre-LVAD than the post-LVAD situation. These post-LVAD alterations may contribute to the reduced force generation of the heart. The differences in this association between the BM and collagen fibers may be due to alterations of the BM, but may also be caused by the changes in the ECM[15, 36, 37].
With IHC we showed a significant reduction of type IV collagen immunoreactivity in the BM

after LVAD support. However, laminin was still present in the basement membrane after unloading of the heart. This suggested that although the basement membrane still surrounds the cardiomyocytes, its structure is severely disorganized. With Q-PCR type IV collagen α1-α6 mRNA expression showed no significant difference before and after LVAD support. Compared to the control group type IV collagen α4 mRNA both pre- and post-LVAD was significantly reduced. Furthermore, compared to the control group a significant increase of the α3 chain and a significant decrease of the α6 chain was observed. So, in heart failure and after LVAD support the composition of the BM may be slightly different from the normal BM. However, our data indicated that the type IV collagen reduction after LVAD support is not due to a completely reduced synthesis of type IV collagen, and must at least in part be the result of an increased degradation by MMP activity. To find out whether MMPs were involved, we investigated 23 different MMP and TIMP in heart biopsies from 8 patients before and after LVAD support with a MMP macro-array and confirmed these measurements with Q-PCR. From these array studies only MMP-2 showed a significant increased ($p < 0.01$) expression after LVAD support. Others have found also other MMP's to be involved in the process of reverse remodeling i.e. MMP-9 [36], but these could not be confirmed in this study. It is known that MMP-2 is able to degrade type IV collagen, but not laminin, in the BM [33, 34, 38]. Falk et al [33] has demonstrated a correlation between the up-regulation of MMP-2 and the degradation of the BM. So, MMP-2 is able to degrade type IV collagen and therefore, can play a major role in remodeling of the BM. The role of MMP-2 before and after LVAD support was therefore, studied in more detail. First the MMP production sites were investigated. With MMP-2 in situ hybridization / IHC we showed that CD3+ cells, macrophages and the endothelium of blood vessels stained positive for MMP-2 mRNA. IHC studies also indicated that MMP-2 protein was located in the sarcoplasm of the cardiomyocytes, infiltrating cells and the endothelium of blood vessels. This indicated that MMP-2 can be produced in the heart by various cell types.

MMP-2 Western blotting showed a low level of active MMP-2 protein pre-LVAD compared to the healthy control. After LVAD support the level of active MMP returns to normal. Gel zymography showed some MMP-2 activity in normal hearts. However, after LVAD support MMP activity was significantly increased compared to pre-LVAD. So, both Western blotting and gel zymography showed an increase in active MMP. This change in active MMP is confirmed by others in heart failure [39, 40] and during LVAD support [36] by gel zymography. Western blot and gel zymography differed in the detected amount of inactive MMP. This may be explained by the difference in sensitivity of the methods, as described by Masure et al [41] and Descamps et al [42]. In normal hearts active MMP was present and it is suggested that this is necessary for a normal collagen turnover in the heart. However, the detected increased levels of active MMP post-LVAD can lead to alterations in the BM membrane. Type IV collagen ISZ showed that this active MMP was present in the BM of the cardiomyocyte before and after LVAD support. Combining the gel zymography and ISZ data, it is likely that

the active MMP-2 is at least in part responsible for the degradation of the BM during LVAD support, although we cannot exclude that other enzymes are involved in the BM breakdown as well.

Although there was a wide variation in the duration of LVAD support (35-557 days), no differences in the reduction of type IV collagen immunoreactivity and MMP-2 localization or activity have been detected between patients supported for a short period or for a long period of time. This suggests that the type IV collagen breakdown in the BM by MMP occurs in the first period after LVAD support and the heart is not able to restore the BM properly. These findings support our previous results, that indicated that the decrease in size of the cardiomyocytes occurred within the first 100 days after LVAD implantation (15).

In conclusion, LVAD support leads to reverse remodeling, including the decrease of cardiomyocyte size. Although the BM becomes more regular after LVAD support as seen by TEM, type IV collagen is not or only weakly detectable in the BM, probably due to increased MMP-2 activity. Furthermore disturbed connections between the BM and the collagen fibers in the ECM were observed by TEM after LVAD support. The disturbed linkage between the BM and the collagen fibers in the ECM may lead to impaired force transmission. This could be one of the reasons why weaning of the LVAD in patients with severe heart failure is only possible in a minority of patients. It may be speculated that restructuring of the BM requires the stimulus of cardiac reloading.

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6

Brain Natriuretic Peptide is produced both by cardiomyocytes, and cells infiltrating the heart in patients with severe heart failure supported by a Left Ventricular Assist Device

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Abstract

Background: B-type natriuretic peptide (BNP) is a cardiac neurohormone synthesized in cardiac ventricles as a result of increased wall stress. Left Ventricular Assist Device (LVAD) support in patients with end stage heart failure results in reduced wall stress and therefore may change BNP levels in the heart

Methods and results: BNP plasma levels were measured in 17 patients with end-stage HF before LVAD implantation and at 1 week, 1 month and 3 months after LVAD support. BNP-mRNA expression in cardiac biopsies of 27 patients before and after LVAD support was determined by quantitative PCR. Immunohistochemistry (IHC) and IHC-double staining was used in 32 patients pre- and post-LVAD support to localize the BNP protein expression in the heart. BNP plasma levels significantly decreased from 1,872 ± 1,098 pg/ml pre-implantation, to 117 ± 91 pg/ml at 3 months after implantation of the LVAD. This decrease in plasma levels was accompanied by a significant decrease in mRNA expression (relative quantity) in the heart. IHC and IHC-double staining showed BNP immunoreactivity in the cardiomyocytes, endothelial cells, infiltrating T-cells and macrophages.

Conclusion: The significant decrease in serum BNP concentration after LVAD support coincides with a decrease in BNP mRNA and protein expression in the heart. BNP is produced in the left ventricle not only by cardiomyocytes, but also by endothelial cells, T-cells, and macrophages. Unloading of the LV by LVAD results in decreased BNP expression in the heart and plasma, and may play an important role in the reverse remodeling process of the heart.

Introduction

Brain Natriuretic Peptide (BNP) is a member of the natriuretic peptide family. It is mainly synthesized and secreted in the cardiac ventricle [1]. BNP is released as preproBNP, which is cleaved into proBNP and subsequently cleaved into the active hormone BNP [2]. BNP is known to have natriuretic, diuretic and vasorelaxant properties and may have antagonistic effects on the renin-angiotensin-aldosteron system [3]. Patients with congestive heart failure have an increased BNP plasma concentration. This is of prognostic value as a marker of morbidity and mortality [4-8]. During the early phase of acute myocardial infarction, plasma BNP levels are strongly increased as well [9]. This is suggestive of an important role for BNP in the process of tissue remodeling. It has been demonstrated that BNP exhibits an anti-fibrotic effect by decreasing collagen synthesis and increasing matrix metalloproteinase (MMP) production [10]. Recently, the neutrophil infiltration in the heart after myocardial infarction has also partially been ascribed to increased BNP levels [11]. During heart failure there is also an increase of infiltrating cells in the heart. T-cells are known to synthesize and express MMP and different cytokines and are therefore known to play an important role in the process of remodeling [12]. These cells may well be an additional source of BNP.

Left ventricular assist devices (LVAD) are commonly used in patients with heart failure as a bridge to heart transplantation (HTx). In most cases, LVAD support extends the patient's life span and improves the quality of life [13-15]. In addition, pressure and volume unloading of the left ventricle, provided by the LVAD, can reverse left ventricular dilatation (reverse remodeling) and lead to regression of left ventricular hypertrophy and neurohormonal changes [16, 17]. Several studies [18-20] indicated a decreased BNP plasma concentration after LVAD support in patients with end stage heart failure. The level of decrease depended on the type of LVAD used.

The aim of the present study was to investigate whether changes in BNP mRNA levels in the heart coincided with changes in BNP plasma levels during LVAD support and which cells in the heart produce BNP. For this purpose BNP plasma concentrations at different time points were measured. Micro laser tissue dissection and quantitative PCR were used to selectively analyze BNP mRNA expression in both cardiomyocytes and CD45+ infiltrating cells in the heart. Immunohistochemistry (IHC) and immunohistochemical double staining was performed to localize and characterize BNP producing cells in the heart.

Materials and methods

Patients

Thirty-two patients (table 1) were enrolled in this study (age: 40 ±14 years; 29 men and 3 women) with refractory end-stage heart failure. Thirteen patients were diagnosed with ischaemic heart disease (IHD) and 19 patients suffered from dilated cardiomyopathy (DCM).

They were treated with a pneumatic or electric LVAD (Heart-mate, Thoratec, Pleasanton, California) as a bridge to heart transplantation (HTx) between March 1993 and November 2003. Twenty-eight of these patients were successfully transplanted. Four patients died before HTx, two as a result of recurrent cerebral embolism, one patient died due to chronic ischaemic enteritis, and one because of an urosepsis. The mean duration of LV unloading was 191 ± 111 days (range 13-455 days). At the time of LVAD implantation all patients were in NYHA functional class IV and in NYHA functional class I while on LVAD support. Informed consent was obtained from all patients. Myocardial biopsies at the time of LVAD implantation were taken from the LV apical core, which was removed during Heart-mate implantation. These biopsies (pre-LVAD) were compared with LV tissue specimens of the explanted heart after HTx (post-LVAD), from the apical half of the LV, outside the suture area of the inflow canula. In the four patients that died, tissue specimens of the heart were obtained at autopsy. All biopsies were directly fixed in buffered formalin and embedded in paraffin. Furthermore, in 27 cases tissue was directly frozen after implanting of the LVAD and after HTx. Complete series of blood samples of 17 patients was collected by venapuncture in EDTA tubes. Blood samples were taken just before LVAD implantation and at 1 week, 1 month, and 3 months after implantation.

Table 1. Characteristics of LVAD patients at time of implantation (n = 32)

Male / Female	29 / 3
Age (yr.)	40 ± 14
DCM / IHD	19 / 13
LVEF (%)	15 ± 5
CO (L/min/m^2)	3 ± 0.8
MAP (mm Hg)	62 ± 8
PVR (dyne sec cm^{-5})	206 ± 75
Mean duration support	191 ± 111

DCM, dialated cardiomyopathy; IHD, ischemic heart disease; LVEF, left ventricular ejection fraction; MAP, mean arterial pressure; PVR, pulmonary vascular resistance

Cross-sectional area of the cardiomyocytes.

The cardiomyocyte cross-sectional area was measured as described by De Jonge et al [21]

Quantitative PCR for BNP.

To determine the BNP mRNA expression in heart tissue, RNA was isolated from frozen tissue sections using TRIzolTM Reagent (GibcoBRL, Rockville, USA). Three μg of RNA was taken for cDNA synthesis using oligo-dT and random primers. Five μl of a 1:20 dilution of the cDNA was used in the Assay-on-Demand kit (Applied Biosystems, CA, USA) for quantitative PCR (Taqman P7700, Applied Biosystems). Total volume was 25 μl.

To determine the BNP mRNA expression in infiltrating cells and cardiomyocytes, mRNA was isolated from different areas in heart tissue after immunohistochemical staining. Frozen tissue sections were stained for CD45 by a standard two-step immunohistochemical procedure.

Primary antibody used was CD45 (polyclonal 1:100; ITK diagnostics). Powervision anti rabbit was used as secondary antibody. After laser micro dissection (P.A.L.M. Mikrolaser, Bernried, Germany) of areas with CD45+ cells, cardiomyocytes and ECM devoid of CD45+ cells, mRNA was isolated using magnetic oligo-dT beads (GenoprepTM mRNA beads, Qiagen, Venlo, The Netherlands) and cDNA synthesis was performed by using oligo-dT and random primers. All cDNA was used in the Assay on Demand kit for the quantitative PCR for BNP. Total volume was 25μl. The following thermocycle profile was used for BNP, PBGD and 18S (house keeping genes): 95°C for 10 min followed by 45 cycles of 95°C for 15 sec and 60°C for 1 min. Each sample was run in duplicate. The following formula was used to quantify the amount of mRNA in our samples: relative quantity = $2^{-\Delta\Delta Ct}$,, $\Delta\Delta Ct = \Delta Ct$ (sample) - ΔCt (calibrator) and ΔCt = Ct (target) – Ct (reference). The calibrator sample was mRNA isolated from human placenta tissue.

BNP immunohistochemistry.

To study the BNP localization in myocardial tissue sections a two-step immunohistochemical analysis was performed on paraffin embedded biopsies. Pepsin was used as antigen retrieval system. After an endogenous peroxidase block myocardial tissue sections were pre-absorbed using 10% normal swine serum (NSS) for 30 min and incubated with the primary antibody rabbit anti human BNP (1:300; Peninsula Laboratories, inc, California) in 10% NSS in PBS for 1 h. Then, sections were incubated with rabbit powervision for 30 min. After development of the peroxidase with diaminobenzidine (DAB)/0.03% H_2O_2, the tissue sections were counterstained with Haematoxylin, dehydrated, and embedded. Omission of the primary antibody and replacement of the primary antibody by isotype-matched irrelevant antibodies served as negative controls. Only results that stood up to these controls are presented.

All slides were examined by two investigators in a blinded manner. The histological findings were described and the immunohistochemical staining graded, as 0 = negative, 1 weakly positive, 2 = positive, 3 = strongly positive. Values for each slide were obtained by taking the mean of all scored areas.

Double-immunofluorescence staining

To investigate which type of infiltrating cells showed positive staining for BNP, the following double-immunofluorescent stainings were performed: BNP-CD3 (T-cells), BNP-CD45 (leucocytes), and BNP-CD68 (macrophages) using tyramide-amplification, as described by van Hoffen et al [22]. After deparaffination the sections were treated with antigen retrieval solution. Primary antibodies used were BNP (1:400; Peninsula Laboratories), CD45 (monoclonal, 1:100), CD3 (monoclonal, 1:100; both ITK diagnostics, Uithoorn, The Netherlands), and CD68 (monoclonal, 1:800; Novocastra laboratories Ltd, UK). As secondary antibody for BNP powervision anti-rabbit (Klinipath, Duiven, The Netherlands) and for CD45, CD3 and CD68 powervision anti-mouse (Klinipath) were used.

BNP plasma levels

Blood samples were collected just before LVAD implantation and 1 week, 1 month, and 3 months after LVAD implantation, centrifuged and plasma was stored at –135°C until use. BNP was analyzed on an ADVIA Centaur immunochemistry system (Bayer diagnostics, Tarrytown, NY).

Statistical analysis

The Q-PCR data, immunohistochemical staining and BNP plasma concentration data were compared by using the Wilcoxon signed rank test. All data were calculated with the statistical package of Prism 3.02 for Windows. A p value < 0.05 was considered significant.

Results

Cross sectional area of the cardiomyocytes

A previous study of our group [21] has shown that the average cardiomyocyte cross sectional area decreased significantly form 519 ± 94 μm2 to 319 ± 53 μm2 after LVAD support, a reduction of 36 ± 19 %. Normal values in five control-patients showed a cross-sectional area of 226 ± 67 μm2.

Quantitative PCR for BNP mRNA in heart tissue.

Quantitative PCR for BNP mRNA was performed on frozen myocardial biopsies of patients before and after LVAD implantation. The relative abundance of the BNP mRNA expression in total heart tissue was calculated compared with a calibrator sample. Almost all (22/27) patients showed a significant decrease of BNP mRNA expression after LVAD implantation. The mean amount of mRNA decreases with about 67% Post-LVAD (p< 0.001, figure 1).

After laser micro dissection of cardiomyocytes and an area with infiltrate containing mainly CD45+ lymphocytes in heart tissues of 3 patients pre- and post- LVAD support (Figure 2), we detected BNP mRNA expression in all samples. The mRNA expression in both the cardiomyocytes and CD45+ cells post-LVAD were decreased in two patients and increased in 1 patient compared to pre-LVAD support.

Figure 1.

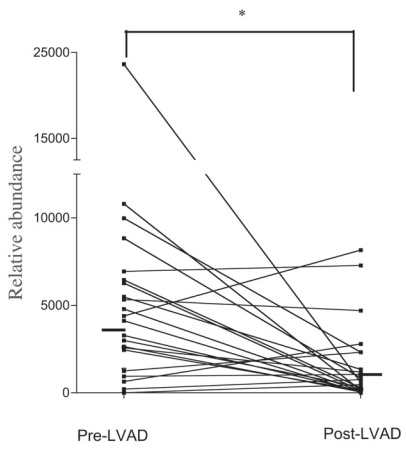

Figure 1. BNP mRNA expression in frozen (total) heart biopsies of heart failure patients before and after LVAD support. The mean mRNA amount decreases with 67% * p< 0.001.

We can not totally exclude that apart from $CD45^+$ leucocytes, other cell e.g. fibroblasts from the ECM have been present in the micro dissected material and contributed to the BNP mRNA expression found in these areas. However, these findings indicate that BNP mRNA is expressed not only in cardiomyocytes but also in other cells.
To test whether mononuclear cell can produce BNP, Peripheral Blood Mononuclear Cells (PBMC) from the same patients were tested in a stimulation assay. After stimulation of the PBMC with TNFα a weak BNP mRNA expression was observed (mean relative abundance of BNP produced by T-cells post-LVAD (n=3): 4.9 ± 3.0; the mean relative abundance of BNP produced by myocardial biopsies post-LVAD: 1,316 ± 2,150).

Immunohistochemical localization of BNP production in heart tissue

IHC staining for BNP was performed on paraffin embedded myocardial tissue sections from patients before and after LVAD support. Cardiomyocytes pre- and post-LVAD support showed sarcoplasmic staining for BNP. But also the endothelial layer of the blood vessels and some cells infiltrating the heart showed positive immunohistochemical staining for BNP (Figure 3 and 4). Grading of the BNP staining in these biopsies showed a significantly reduced sarcoplasmic staining post-LVAD in comparison to pre-LVAD (2.2 ± 0.7 pre vs. 1.7 ± 0.7 post, $p < 0.001$; figure 3). However, BNP reactivity in infiltrating cells was present equally pre- and post-LVAD and the number of immunohistochemically BNP positive T-cells appeared to be the same before and after LVAD support. Double staining showed that positive immunoreactivity for BNP was present in CD45, CD3 and CD68 positive cells. Data for T-lymphocytes are shown in figure 4.

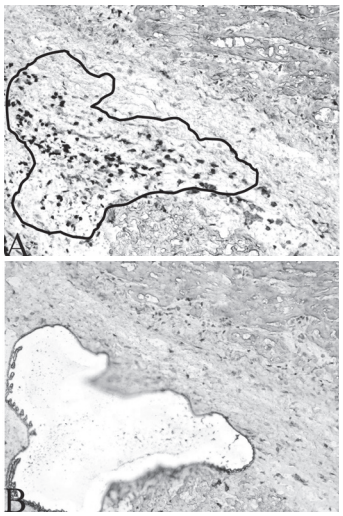

Figure 2. Laser micro tissue dissection of an area containing CD45+ infiltrating cells in frozen myocardial biopsies. Immunohistochemical staining with anti CD45 antibody (A). An area containing many CD45+ cells is micro dissected (B) and used for Q-PCR of BNP mRNA.

BNP plasma levels pre- and post-LVAD

BNP ELISA was performed in plasma samples one week, one month and three months after LVAD support. As shown in table 2, all 17 patients had high levels of BNP before LVAD implantation. One week after implantation of the LVAD the BNP levels decreased significantly. Between one week and 1 month after implantation BNP seemed to stabilize followed by a further decrease between 1 month and 3 months after implantation.

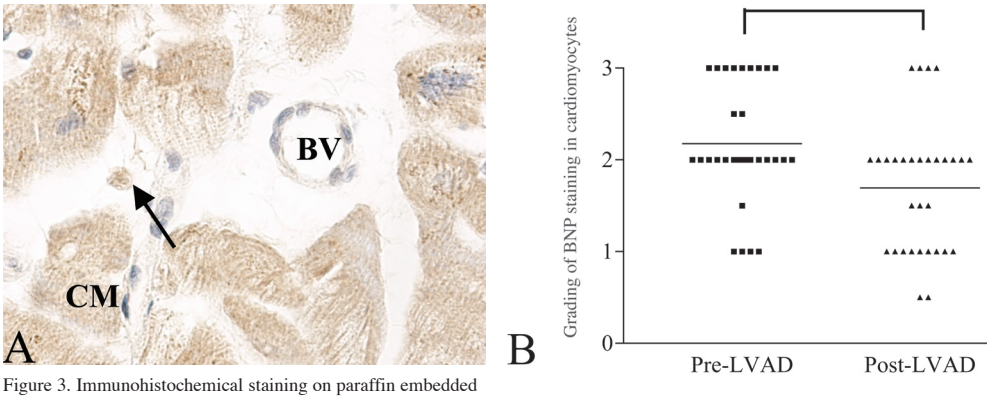

Figure 3. Immunohistochemical staining on paraffin embedded heart biopsies for BNP.
Positive immunoreactivity in cardiomyocytes and the endothelial layer of the blood vessel (A). Changes in the immunoreactivity in the cardiomyocytes before and after LVAD support as graded on a scale of 0 to 4 (B). * p< 0.001. CM = cardiomyocyte, BV = blood vessel, Arrow = infiltrating cell

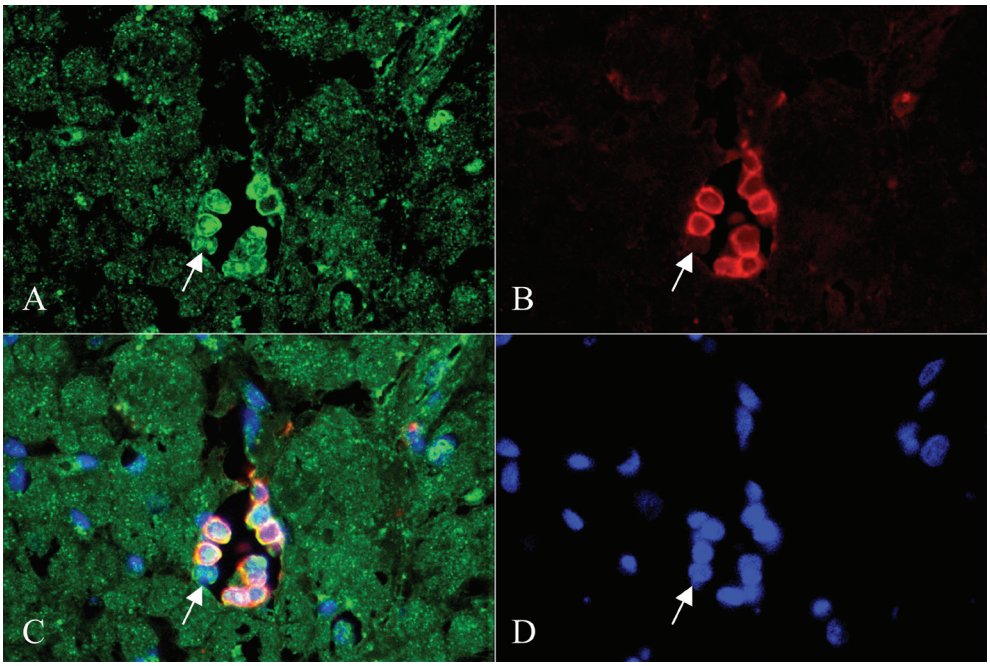

Figure 4. BNP immunoreactivity in T-cells in myocardial biopsies pre-LVAD.
A: BNP expression in infiltrating cells in green.
B: CD3 expression in infiltrating cells in red.
C: Double staining for BNP (green) and CD3 (red).
D: Nuclear (DAPI) staining (blue).
Arrow: CD3- BNP+ cell

Discussion

This study showed that BNP plasma levels were high in patients with congestive heart failure accompanied by high BNP mRNA and BNP protein expression in the heart. BNP mRNA, and BNP immunoreactivity were detected in the cardiomyocytes, and also in infiltrating cells. After LVAD support, both mRNA expression and protein expression significantly decreased in the cardiomyocytes, in parallel with decreased BNP plasma levels in these patients.

BNP is a cardiac neurohormone secreted from the ventricles in response to cardiomyocyte stress and stretch [6]. Increased plasma BNP levels correlated with dilatation of the LV, decreased LV contractility and ventricular stiffness [11]. Recently, neutrophil infiltration in the heart after myocardial infarction has also partially been ascribed to increased BNP levels [11]. In patients with congestive heart failure infiltrating cells are found in the heart. It is known that T-cells are able to synthesize and secrete different MMP, cytokines and their receptors. The fact that the endocrine and the immune system share common signal molecules and receptors suggests a close interaction between these systems [23] Unloading of the heart by a LVAD leads to reversal of LV dilatation, and to regression of LV myocyte hypertrophy (reverse remodeling)[13, 16, 21, 24].
The aim of this study was to investigate whether BNP mRNA levels in the heart coincide with BNP plasma levels during LVAD support and which cells in heart produce BNP during LVAD support. The reduction of the cross sectional area of the cardiomyocyt after LVAD support showed that LVAD support reduced the stretch of the cardiomyocyt and therefore may have profound effect on the BNP production by the cardiomyocytes. Using Q-PCR, we demonstrated a significant decrease of BNP mRNA in large myocardial biopsies after LVAD support. This was also shown by Kuhn et al [25]. BNP is predominantly synthesized in the cardiac ventricles by cardiomyocytes, but several data suggested that other cells such as cardiac fibroblasts and bone marrow-derived macrophages [10, 26, 27] are also capable of producing BNP. In patients with end-stage heart failure, but also after LVAD support, we could demonstrate that apart from cardiomyocytes some T-cells and macrophages infiltrating the heart display positive immunoreactivity for BNP. This positive immunoreactivity of the infiltrating cells (T-cells and macrophages) was confirmed by double staining of BNP with CD3 and CD68. Furthermore, after laser micro tissue dissection of intra myocardial $CD45^+$ cells in the heart, mRNA for BNP was detected in these cells. This indicated that BNP is actively synthesized and secreted by T-cells and/or macrophages. Theoretically, macrophages could display immunoreactivity by phagocytosis of BNP, but Vollmar [26] et al have shown that BNP was actively produced by peritoneal and bone marrow-derived macrophages in mice [26]. In this study we observed a weak BNP mRNA expression after stimulation of mononuclear cells with TNFα. This indicates that lymphocytes are able to produce BNP mRNA after stimulation with a proinflammatory cytokine. As in heart failure often high levels of TNFα are detected [28] TNFα in combination with the microenvironment in the heart is probably important for

the induction of BNP in mononuclear cells. The role of proinflammatory cytokines in stimulation of BNP expression was also investigated by Ma et al [29]. They showed that TNFα was able to selectively stimulate BNP mRNA and BNP secretion in cardiocyte cultures. After LVAD support the BNP immunoreactivity in the heart decreased in cardiomyocytes, which was paralleled by a decrease of BNP mRNA expression and a decrease of BNP plasma concentration. However, the number of BNP positive immunohistochemically T-cells appeared to be the same before and after LVAD support. These findings may also suggest that the microenvironment in the heart during heart failure and LVAD support stimulates T-cells not only to produce different MMP and cytokines but also BNP. In acute cellular rejection after heart transplantation an increase of plasma BNP and myocardial BNP mRNA has been demonstrated [30-32]. It is suggested that pro-inflammatory cytokines produced by infiltrating T-cells in this situation are responsible for stimulating cardiomyocytes to secrete BNP. In line with our findings, these infiltrating cells themselves may be responsible, at least in part, for the increased BNP production and plasma levels. This is supported by the finding that T-cell depletion in the heart as anti-rejection therapy [30], leads to decreased BNP plasma levels. So, BNP plays an important role in remodeling of the heart by decreasing fibrosis [10] but may also be involved in the modulation of immunological processes locally in the heart. This is the first study demonstrating BNP production by T-lymphocytes and macrophages locally in the heart in patients with end-stage heart failure. The exact role of this BNP production and its consequences for the immune system have to be elucidated.

In conclusion, LVAD support leads to decreased wall stress and stretch by mechanical unloading. This unloading results in a decrease of cardiomyocyte size and in BNP mRNA and protein expression in the heart and a decrease in plasma levels in patients after LVAD implantation. In addition, BNP is not only produced by cardiomyocytes but also by individual T-cells and macrophages localized in the heart in severe heart failure and after LVAD support.

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The Role of Cathepsin K in Extracellular Matrix remodeling in patients with end stage heart failure.

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Abstract

Background: Alterations in the collagen structure and composition of the extracellular matrix (ECM) in the myocardium (remodeling) influence left ventricular geometry and therefore, play an important role in development of end-stage heart failure. Although it is known that matrix metalloproteinases (MMP) are important in ECM remodeling, it is not excluded that other proteolytic enzymes play an important role in these collagen turnover as well. Therefore, we studied the role of cathepsin K in patients with end stage heart failure both before and after Left Ventricular Assist Device (LVAD) support.

Methods and Results: Cathepsin K mRNA expression was significantly higher before LVAD support compared to the healthy control. After LVAD support, cathepsin K mRNA was even more significantly increased compared to pre-LVAD and the healthy control. Likewise, cathepsin K Western blotting detected a higher level of cathepsin K protein in patients with end-stage heart failure compared to healthy controls. However, after LVAD support the protein level decreased. Immuno histochemical analysis showed that the macrophages in the heart are the main producers of cathepsin K. The number of macrophages decreased after LVAD support.

Conclusion: In patients with end stage heart failure cathepsin K is expressed by macrophages and may contribute to ECM remodeling in the heart. During unloading of the heart by a LVAD, the number of macrophages decreased resulting in a decreased level of cathepsin K. This suggested that cathepsin K is more important in the process of remodeling during heart failure than in the process of reverse remodeling during LVAD support.

Introduction

Structural remodeling in heart failure is characterized by rearrangement of the architecture of the cardiac ventricular wall. It involves hypertrophy of the myocytes, fibroblast proliferation, and increased deposition of extracellular matrix (ECM) proteins[1]. The major components of the ECM are the fibrillar collagens (type I and III). These collagens ensure structural integrity of adjoining myocytes and are essential for maintaining alignment of myofibrils within the myocyte through a collagen-integrin-cytoskeletal-myofibril relation[2-5]. The development of left ventricular (LV) dilation in patients with congestive heart failure (CHF) has been shown to be associated with discontinuities of the fibrillar collagen network. Furthermore, in a previous study of our group [6] increased collagen degradation products have been measured in the plasma of patients with CHF. Matrix metalloproteinases (MMPs) have been extensively studied for their role in ECM turnover/degradation in heart failure and after LAVD support[7]. Although several groups did find increased levels of the gelatinases (MMP-2 and MMP-9), the role of the collagenase (MMP-1, MMP-8, and MMP-13) in ECM remodeling in the heart is not convincing. Cysteine proteases have also been linked with matrix turnover: they degrade existing ECM and limit the release of newly synthesized ECM from fibroblasts. Within the papain-cysteine proteinase family, three proteases show significant matrix-degrading activities, i.e., cathepsin K, cathepsinL, and cathepsinS. Among these, cathepsin K been shown to possess a unique collagenolytic activity[8, 9]. This activity does not depend on destabilization of the triple helix, but rather it cleaves type I and II collagen at the ends (telopeptide) and at multiple sites within the native triple helix[10]. The role of cathepsin K in bone turnover is well established, however, evidence is increasing that cathepsin K is also involved in pathological processes unrelated to bone remodeling, e.g., granulomatous diseases, amyloidosis, and atherosclerosis[11-14]. Left ventricular assist devices (LVAD) are used as bridge to heart transplantation in patients suffering from CHF. As described [6, 15] LVAD support induces reverse left ventricular remodeling leading to alterations in the ECM and sometimes partial recovery of ventricular functions. To the best of our knowledge, there have been no published reports on cathepsin K in relation to patients with CHF or after LVAD support. The aim of this study is to investigate if cathepsin K plays a role in ECM remodeling and reverse remodeling in patients with CHF before and after LVAD support. Expression of cathepsin K was determined on mRNA level with Quantitative PCR, and on the protein level with Western Blot analysis and Immunohistochemistry (IHC).

Methods

Patients and materials

In this study 14 patients (mean age: 49 ±12 years; 12 men and 2 women) were included with refractory end-stage heart failure. All were treated with a LVAD (Heart-mate, Thoratec,

Pleasanton, California) as a bridge to transplantation. All patients were successfully transplanted. The mean duration of LV unloading was 181 ± 112 days (range 10-334 days). All patients were in NYHA class IV at the time of LVAD implantation, and in NYHA class I while on LVAD support. Informed consent to participate in this study was obtained from all patients. Myocardial tissue was taken at time of LVAD implantation from the LV apical core. These biopsies (pre-LVAD) were compared with LV tissue specimens of the explanted hearts after HTx (post-LVAD) from the apical half of the LV, outside the suture area of the inflow canula. All biopsies were directly fixed in buffered formalin and embedded in paraffin or were frozen in liquid nitrogen. Control tissue was taken from the left ventricle of non-used donor hearts (n=5).

Quantitative PCR for Cathepsin K

To determine the mRNA expression of cathepsin K in heart tissue, RNA was isolated from frozen heart tissue sections (before and after LVAD support) using TRIzolTM Reagent (GibcoBRL, Rockville, USA). Three μg of RNA was used for cDNA synthesis using oligo-dT and random primers. Five μl of a 1:5 dilution of the cDNA was used in the Assay-on-Demand kits for cathepsin K (Applied Biosystems, CA, USA) for Q-PCR (Taqman P7700, Applied Biosystems). Porphobilinogen deaminase (PBGD) was chosen as reference gene.

Western blotting

Immunoblotting for cathepsin K was performed on proteins isolated from myocardial biopsies before and after LVAD support (n = 14). Equal amounts of protein were separated on a 12% precast SDS PAGE gel (Bio-Rad Laboratories, Veenendaal, The Netherlands) and transferred to a nitrocellulose membrane. Western blot analysis was performed with a mouse monoclonal anti-cathepsin K antibody (1:500; Novocastra, Newcastle upon Tyne, UK) in PBS containing 5% milk. After incubation with RAMPO as secondary antibody, the blot was developed using the Amersham ECL system (Amersham Pharmacia Biotech Europe GmbH).
Tubulin was used as loading control. The intensity of the bands was quantified with the computer program TotallabV 2003.03 (Nonlinear Dynamics, Newcastle upon Tyne, UK).

Immunohistochemistry of Cathepsin K and CD68

To localize cathepsin K protein and determine the number of CD68 positive macrophages in the heart, a three-step immunohistochemical analysis was performed [15, 16] on paraffin sections. Primary antibodies used were: monoclonal antibody anti-cathepsin K (1:100) and polyclonal antibody anti-CD68 (1:800, both Novocastra). Subsequently, the slides were incubated with rabbit anti mouse peroxidase (RAMPO; 1:250) and rabbit powervision (Klinipath, Duiven, The Netherlands). Citrate boiling was used as antigen retrieval. The specificity of the IHC was confirmed by positive and negative controls. Tissue of a giant cell sarcoma was used as positive control. Omission of the primary antibody and replacement of the primary antibody by isotype-matched irrelevant antibody served as negative controls.

Only results that stand up to these controls are presented. All slides were examined by two investigators in a random blinded manner. The staining intensity was graded as: 0 = negative, 1 = weakly positive, 2 = positive, 3 = strongly positive, 4 = very strongly positive. The number of macrophages in the myocardium and endocardium were scored as: 0 no macrophages observed, 1 = some macrophages observed, 2 = more macrophages observed, 3 = lots of macrophages observed. Values for each slide were obtained by taking the mean of all scored areas.

Statistical analysis

Grading of the IHC, Q-PCR data, and WB measurements were compared using the paired Wilcoxon signed rank test or the Mann Whitney test. Inter- and intra- observer variability was tested using kappa analysis. All data were calculated with the statistical package of Prism 3.02. A p value < 0.05 was considered significant.

Results

Increased Cathepsin K mRNA levels after LVAD support.

To determine the cathepsin K mRNA levels in the heart biopsies, Q-PCR was performed on mRNA isolated from heart tissue of 14 patients before and after LVAD support. Before LVAD support significantly higher levels of cathepsin K were observed compared to the healthy control ($p = 0.05$) After LVAD support mRNA levels were significantly increased by 63% compared to the pre-LVAD situation (figure 1; $p<0.01$).

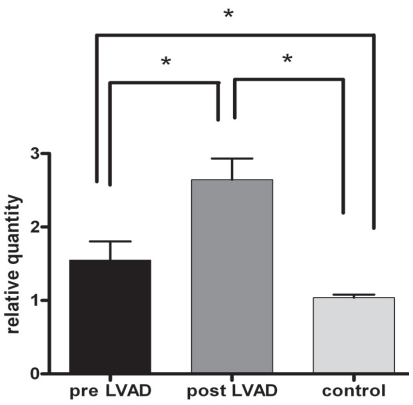

Figure 1. Cathepsin K mRNA expression pre- and post-LVAD support.
Cathepsin K mRNA expression before LVAD support was significantly higher compared to healthy control ($p = 0.05$). Cathepsin K mRNA expression was significant increased by 63% after LVAD support ($p < 0.01$) compared to pre LVAD and healthy control (* $p < 0.01$).

Cathepsin K protein in heart tissue pre- and post-LVAD

Western blotting

To quantify the cathepsin K protein expression in heart tissue, proteins isolated from frozen heart tissue of 14 patients pre- and post-LVAD was analyzed by Western blotting. Total cathepsin K did not significantly change pre-LVAD compared to post-LVAD. However, the active form of cathepsin K (represented by the 28 kDa band) decreased significantly after LVAD support by 30% as compared to pre-LVAD ($p= 0.03$; figure 2). The inactive form (as represented by the 37 kDa band) did not change significantly. Cathepsin K protein levels are significantly higher both in pre- and post LVAD tissues than in control heart tissues.

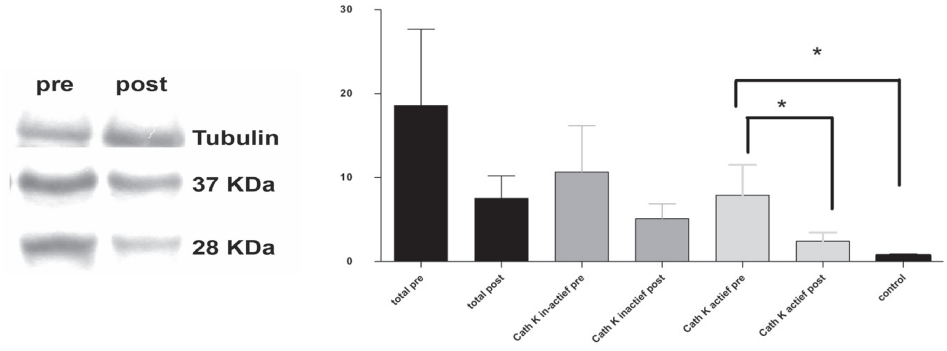

Figure 2. A: A representative immunoblot stained with anti-cathepsin K in myocardial biopsies pre- and post-LVAD support. The 37 kDa band represents inactive cathepsin K and the 28 kDA band represents active cathepsin K. Tubulin was used as loading control
B: Quantification of the immunoblot, as presented in the bar graph, showing a significant higher level of active cathepsin K pre LVAD compared to post LVAD and the healthy control (n = 14; * p = 0.03).

Immunohistochemistry (IHC)

To localize cathepsin K protein and to determine the number of macrophages in heart tissues of patients before and after LVAD, IHC for cathepsin K and CD68 was performed on 14 patients before and after LAVD support. Cathepsin K protein expression was mainly localized in the macrophages in the endocardium and sometimes in the myocardium (figure 3). Cardiomyocytes did not stain positively for cathepsin K. Semi quantitative grading using a 0-4 grading scale showed that before LVAD support the number of macrophages and the intensity of the cathepsin K staining in the heart biopsies was significantly higher compared to post LVAD (p = 0.04 and 0.02) The number of macrophages as determined by CD68 expression, decreased after LVAD support by 50%, from 2.3 to 1.1.

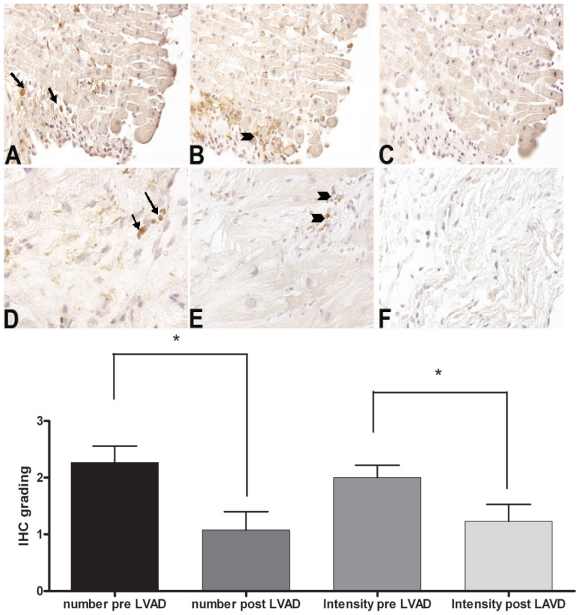

Figure 3. Cathpsin K and CD68 (macrophages) immunohistochemical staining in heart tissue.
Cathepsin K: both in pre- (A) and post- (D) LVAD support myocardial tissue showing positive immunoreactivity for cathepsin K. No staining was observed in the cardiomyocytes and the ECM. C. Negative control. No staining was observed. Magnification 200x. Arrow = cathepsin K positive cells. IHC staining for CD68 both in heart tissue pre- (B) and post (E) LVAD support, indicating that macrophages were present in the heart biopsies. F negative control. No staining was observed.. Arrowhead = CD68 positive cells in the heart.
G. Quantification of the number of macrophages and the intensity of the cathepsin K staining in these macrophages in the heart. Post LVAD the number of macrophages and intensity of cathepsin K staining in the macrophages was significantly decreased compared to pre LVAD (p = 0.02 and 0.04, respectively).

Discussion

Heart failure is characterized by ECM remodeling resulting from the imbalance between synthesis and degradation of ECM. While the role of several matrix metalloproteinases and their inhibitors in ECM matrix remodeling is intensively studied, the role of other enzymes that may contribute to ECM remodeling in the heart has still to be elucidated. In this paper the role of cathepsin K in ECM degradation was investigated in patients with congestive heart failure (CHF). We observed that cathepsin K mRNA and protein were strongly expressed in patients with CHF (pre LVAD) compared to healthy controls. The protein expression decreased after LVAD support, this in contrast to mRNA levels that increased post-LVAD. Cathepsin K was expressed in the heart in macrophages. The number of cathepsin K positive macrophages decreased significantly after LVAD support compared to pre-LVAD.

The ECM consists of many components including the fibrillar (type I and type III) collagens and provides anchorage, support and structure to the myocardial tissue. Discontinuities within the fibrillar network or its disruption will leads to the loss of its normal continuity and structural support function. Although MMPs have been shown to play an important role in remodeling of myocardial ECM, there are several studies suggesting that also cysteine proteinases, like cathepsin K may contribute to the cleavage of type I collagen in the ECM [9]. Cathepsin K has strong collagenase, elastase and gelatinase activity and therefore, also possesses the ability of remodelling the ECM, either on its own, or in concert with other collagenolytic and gelatinolytic enzymes[8, 9]. Although tissue expression of cathepsin K is normally quite low outside the bone, the enzyme has been observed is several organs and cell types such as heart, lung, skeletal muscle, colon, placenta and within the context of inflammation [8, 14, 17, 18]. In our study with Q-PCR significantly high levels of cathepsin K mRNA in heart failure tissue compared to normal heart tissue were observed. After LVAD support this mRNA expression increased significantly compared to pre-LVAD. Also with cathepsin K Western blotting significantly higher cathepsin K protein levels were detected in patients with severe end-stage heart failure (pre- and post- LVAD), compared to the healthy control. However, in contrast to mRNA levels the cathepsin K protein level in heart tissue after LVAD support decreased significantly compared to pre-LVAD. With IHC, cathepsin K was shown to be produced by the macrophages in the heart. Quantification of the number of macrophages revealed a decrease in the number of macrophages in the myocardium post-LVAD compared to pre-LVAD. No cathepsin K staining was observed in the cardiomyocytes or in other cells in the ECM. Cathepsin K expression by macrophages is confirmed by various other studies, for example in atherosclerosis [19, 20]. The decrease of cathepsin K protein after LVAD support, was however, not paralleled by a reduced mRNA expression. In fact cathepsin K mRNA levels increased during LVAD support. This discrepancy between the mRNA levels and the protein levels of cathepsin K is most likely due to translational efficiency, or post-transcriptional regulation. In previous studies, we have shown that the ECM in patients with sever end-stage heart failure is actively remodeled. Before LVAD

support high levels of collagen degradation products were observed [6]. However, we did not find increased levels of MMP (collagenase) activity[7]. Only MMP-2 was increased in these patients. Although MMP-2 is able to degrade the fibrillar collagens of the ECM in the heart, it is not likely that MMP-2 alone is responsible for the observed degradation of the ECM. MMP-2 is more specific for gelatinases and type IV collagen. In this study we showed significant high mRNA and protein levels of cathepsin K in patients with severe end stage heart failure before LVAD support. It is therefore likely that cathepsin K play a role in ECM remodeling in patients with end stage heart failure.

In conclusion, cathepsin K is produced by macrophages in the failing human heart and contributes to the ECM remodeling in patients with end stage heart failure. Unloading of the heart results in a decrease of macrophages numbers in the heart and this decreased macrophage numbers result in a decrease of cathepsin K protein levels. This suggests that cathepsin K is more important in remodeling during heart failure, but less important in the process of reverse remodeling during LVAD support.

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8

General discussion

Heart failure is still one of the major causes of death in the western world, despite the improvements and innovations in pharmacologic and surgical therapies. Cardiac transplantation is the only treatment for patients with end-stage heart failure, but due to the shortage of donor organs, only a limited number of patients can be transplanted. Cardiac function can rapidly deteriorate awaiting heart transplantation, and also become resistant to pharmacologic intervention [1]. Unloading the heart by a LVAD in these patients can be life saving. The first aim of the study described in this thesis was to investigate the changes in the ECM during LVAD support, in order to better understand the role of the ECM in heart failure. Secondly, we wanted to answer the question whether LVAD support contributes to the recovery of the LV myocardium, through "reverse remodeling". The results might tell us whether weaning of the LVAD might be feasible in the future.

Previous studies by De Jonge et al in patients on LVAD support demonstrated an increase in exercise capacity, and an improved VO_2 Max after LVAD support [2]. In addition, they observed an improvement of the condition of contractile elements, and a decrease in cardiomyocyte size [3]. These effects are part of the process called reverse remodeling. In spite of these positive effects, unloading the heart by a LVAD does, in a majority of patients, not induce sufficient changes in ventricular function that would permit LVAD removal with sustained cardiac recovery [4, 5]. The only patients with promising outcomes, are those suffering from myocarditis or acute cardiogenic shock [6].

Extracellular matrix remodeling before and after LVAD support

Type I and III collagens

The ECM consists of multiple components of which type I and III collagens are the most abundant. Changes in this fibrillary collagen network can either lead to myocardial rigidity, or to left ventricular dilatation.

In **chapter 4** we studied the ECM volume and fibrillar collagens before and after LVAD support. In patients with heart failure before LVAD support type I collagen degradation was increased (compared to normal hearts), whereas its synthesis was low. It is known from animal studies [7] that an increase in interstitial collagen is associated with diastolic heart failure, whereas degradation of endomysial and perimysial collagens is accompanied by ventricular dilatation and systolic heart failure. Our results, along with the cited data, suggest that the process of remodeling leading to end stage heart failure, is associated with augmented collagen degradation. After LVAD support the opposite occurs. Type I collagen synthesis increases, whereas degradation of type I collagen decreases. The total collagen content did not change however, the structural quality of the collagens was improved, as less denatured (uncoiled) collagen was detected. In heart failure many other ECM components are increased as well, in addition to collagens. These include elastin, fibrillin, fibronectin and proteoglycans [8]. The total ECM volume was also investigated in this thesis. During LVAD support the ECM volume follows a biphasic pattern. The first 100 days after implantation of the assist device the ECM volume increases but it decreases again after prolonged LVAD support. However,

the cardiomyocytes demonstrate already a decrease in size during the first 100 days after the onset of LVAD support. Taken together, these data suggest that during LVAD support the size of the cardiomyocytes and the amount of ECM decrease over time, together with an improvement of the quality of the fibrillar collagens in the ECM. It thus seems that the cardiomyocyte size and fibrillar collagen network tend to normalize after prolonged LVAD support. After LVAD support the normalisation of cardiomyocyte size is a relatively fast process, whereas the ECM needs more time for adaptation.

Type IV collagens

Basement membranes are important structural components of the ECM. The BM connects the cardiomyocyte to the ECM, and therefore plays an important role in transmission of force during the diastole and systole. Disruption of the BM will lead to impaired contractility of the heart. Type IV collagens and laminin are the major components of the BM. A study of the changes in the type IV collagen network was the subject of **chapter 5**.

In healthy control hearts, and in patients with severe end-stage heart failure, the BM is thick and irregular. There are connections between the ECM collagen fibers and the BM. Post-LVAD the BM became more regular and the lamina lucida and lamina densa were more compact and could be better discriminated compared to the healthy control. However, there are fewer connections between the BM and the collagen fibers in the ECM. Furthermore, the type IV collagen surrounding the cardiomyocytes was reduced as revealed by immunohistochemical staining. This effect occurred as early as 35 days after the onset of LVAD support, and was even more prominent after 365 days of LVAD support. This suggests that the structure of the BM, as it presents itself in the normal heart and pre-LVAD, is needed for an adequate connection with the fibrillar collagens in the ECM, and that this structure is severely affected by unloading of the left ventricle with a LVAD. The change in BM morphology and the decrease of type IV collagen as observed in the majority of patients in this study might well be ascribed to a process of atrophy. Muscular atrophy is, in general, can be caused by reduced muscle activity over a prolonged period. Several studies have shown that also in the myocardium ventricular unloading cause atrophy of cardiomyocytes with significant loss of contractile elements and disorientation of sarcomeres within the cardiomyocyte [9, 10]. Such atrophy is to be considered as a physiological response, and is associated with a decrease of cardiac dimension and weight. This atrophy is most likely a reversible process, because the cellular integrity is preserved[11].

Mediators in extracellular matrix remodeling

Under physiological conditions, the ECM is in a state of continuous turnover. This dynamic equilibrium between synthesis and degradation represents a natural balance, comprising *de novo* synthesis and deposition of ECM, and proteolytic degradation of existing ECM. Under pathological conditions, this balance may deteriorate, resulting either in increased or in

decreased amounts of ECM; this dysbalance then progressively contributes to an impairment of cardiac function. In most tissues, degradation of the ECM occurs through the action of matrix metalloproteinases (MMPs) and cysteine endoproteases (cathepsins).

The role of proteolytic enzymes in matrix degradation

The heart contains many MMPs that can degrade ECM proteins. These include two groups of enzymes, the collagenases and the gelatinases. The collagenases (MMP1, MMP8, and MMP13) initiate the ECM degradation process by cleaving the α-chains of type I and type II collagens. The gelatinases (MMP-2 and MMP9) process collagen fragments further. Some MMPs are constitutively expressed and are required for a normal heart function, but the expression of others is regulated, notably by hormones, growth factors, cytokines, and mechanical strain. The activity of MMP is tightly controlled by Tissue Inhibitors of Metalloproteinases (TIMPs). Also expression of TIMPs is tightly controlled during tissue remodeling, in order to maintain the balance in the ECM turnover. Disruption of this balance, i.e. uncontrolled matrix turnover, may result in heart failure.

Our studies (**chapter 4**) have shown an increased degradation of the fibrillar collagens in patients with severe end-stage heart failure before LVAD support. However, there were no significant changes in the expression or the activity of MMP-1, MMP-8, or MMP-13 in these patients. The TIMPs did not change their levels either. In contrast to our results, other investigators have found alterations in MMP-1, MMP-3 and MMP-9 expression during LVAD support [12-14]. The difference in the quantity of MMP expression between their results and those in this thesis may be due to variations in the LVAD support duration in both studies. It is known that MMP-2 is able to degrade the fibrillar collagens as well [15], but it is unlikely that MMP-2 alone is responsible for the degradation of these collagens, as described in chapter 4. The main target of MMP-2 is the gelatines or type IV collagen. We did find high MMP-2 mRNA and protein levels in patients with end-stage heart failure, before LVAD support (**chapter 5**). After LVAD support these levels were even more increased. After in situ zymography we observed high levels of MMP activity surrounding the cardiomyocyte. This strengthens the suggestion that MMP-2 is responsible for the degradation of type IV collagen. Probably other enzymes, which are able to degrade the ECM as well, are involved in the degradation of the fibrillar collagens. Cysteine proteases are able to degrade existing ECM and limit the release of newly synthesized ECM from fibroblasts. Within the papain-cysteine proteinase family, three proteases show significant matrix-degrading activities, i.e., cathepsin K, cathepsin L, and cathepsin S. Among these, cathepsin K has been shown to possess a unique collagenolytic activity. This activity does not depend on destabilization of the triple helix, but rather cleaves type I and II collagen at the ends (telopeptide) and at multiple sites within the native triple helix. The role of cathepsin K in bone turnover is well established, however, evidence is increasing that cathepsin K is involved in pathological processes unrelated to bone remodeling, e.g., granulomatous diseases, amyloidosis, and

atherosclerosis. In **chapter 7** we observed high levels of cathepsin K mRNA and protein in patients with end stage heart failure, before LAVD support. It is therefore likely that not MMP but cathepsin K play an important role in the degradation of the fibrillar collagens in patients with severe end-stage heart failure. However, after LVAD support cathepsin K activity decreases and therefore seems less important in the process of reverse remodeling. In summary we can conclude that the matrix degrading enzymes appear to normalize during LVAD support allowing improvement in quantity and quality of collagen fibers. In contrast, MMP-2 increases during LVAD support and plays an important role in the degradation of type IV collagen in the BM and may therefore be important to sustained cardiac recovery.

Cytokines and BNP

As noted earlier, hormones and cytokines play an important role in the activation of matrix degrading enzymes. Therefore, they may play a role in tissue remodeling during HF and LVAD support as well. In **chapters 1 and 2** we investigated the role of several cytokines and their polymorphisms in patients with severe heart failure, after LVAD support and after heart transplantation. TNFα seems to be the cytokine with a prominent role in the remodeling and reverse remodeling processes. **Chapter 3** showed a significant increase of plasma TNFα levels in patients with end-stage heart failure compared to healthy controls. Patients with severe HF that requires LVAD support produced even more TNFα than patients on medical therapy. During LVAD support TNFα plasma levels decreased significantly. TNFα stimulates, amongst others, BNP secretion [16] suggesting that both factors may be related to the development of heart failure. It is known that TNFα and BNP also play an important role in the regulation of MMP induction [17], but the exact role of BNP in the process of remodeling remains to be elucidated. Stimulation of MMP by BNP may be a compensatory response to prevent excessive collagen deposition. This anti-fibrotic action of BNP may be beneficial to the heart. However, in certain circumstances it is possible that BNP, by increasing MMP activity and consequently ECM degradation, promotes ventricular remodeling leading to dilatation [16]. In **chapter 6** we observed increased levels of circulating BNP in HF. BNP was not only produced by the cardiomyocytes, but also infiltrating T-cells seemed to produce BNP as evidenced by the positive BNP staining of these cells. The stimuli leading to this BNP production in T-cells in the failing heart is still unknown. However, TNFα produced by cardiomyocytes may stimulate the T-cells to express BNP. The elevated BNP serum levels rapidly returned to more normal after LVAD support. This finding strengthens the

Table 1. Summary of the expression of the mediators measured in this thesis.

	Normal heart	Heart failure	After LVAD support
MMP-2	+	++	+++
Cath K	+/-	++	+
TNFα	+	++	+
BNP	+	++	+

All mediators seems to normalize except MMP-2.

notion that the production of BNP is stretch dependent, although it can not ruled out that also hormonal changes contribute to changes in BNP levels. The regulation of both remodeling and reverse remodeling by the various mediators and their influence on ECM producing and degrading components is very complex and require further investigation.

LVAD support: bridge to recovery?

Due to improved technology, increasing clinical experience and expanding indications for their use, LVADs are playing an important role in the treatment of patients with end-stage heart failure [5]. Although the LVAD is used as a bridge to transplantation, it became evident that in some cases the LVAD can be explanted without the further need for transplantation, the so called bridge to recovery. Recently, the group of Birks et al and Hall et al [18, 19] reported promising results in restoring ventricular function in patients with end stage heart failure using a novel combination therapy consisting of a left ventricular assist device (LVAD) combined with pharmacologic therapy including the selective beta(2)-agonist, clenbuterol. However, the number of patients in which this has been successful is low. Several patients have developed heart failure again or died due to heart failure related complications. Therefore presently LVADs in general, are not used with the intention of bridging patients to recovery. Also in our patient group explantation of the LVAD has not been possible. This thesis showed that cardiomyocyte size decreased rapidly after LVAD implantation. The ECM volume decreased also, be it at a much slower rate. Furthermore, during LVAD support the fibrillar collagen structure improved, and BNP and TNFα levels decreased. Taken together, these results suggest that the failing heart normalizes during LVAD support. However, we also observed a decreased immuno staining of the BM surrounding the cardiomyocytes combined with a distorted connection between the BM and the CM during LVAD support. Therefore it seems that during LVAD support the myocardium becomes atrophic.

To reverse this atrophy the heart should be able to contract during LVAD support. Therefore, weaning from the LVAD should ideally be accompanied with a gradual increase in cardiac loading. In this way the heart can adapt to normal exercise. To accomplish this, LVAD support systems are required that can support the heart at different levels of intensity. The LVAD systems used in the present study were not adjustable in respect to cardiac loading.

So, in conclusion during LVAD support, recovery is neither complete (this thesis) nor permanent. Future efforts should therefore concentrate on insights that could result in the development of more effective treatment options for these patients like (stem) cell therapy or drugs specifically counteracting the heart failure related deteriorations

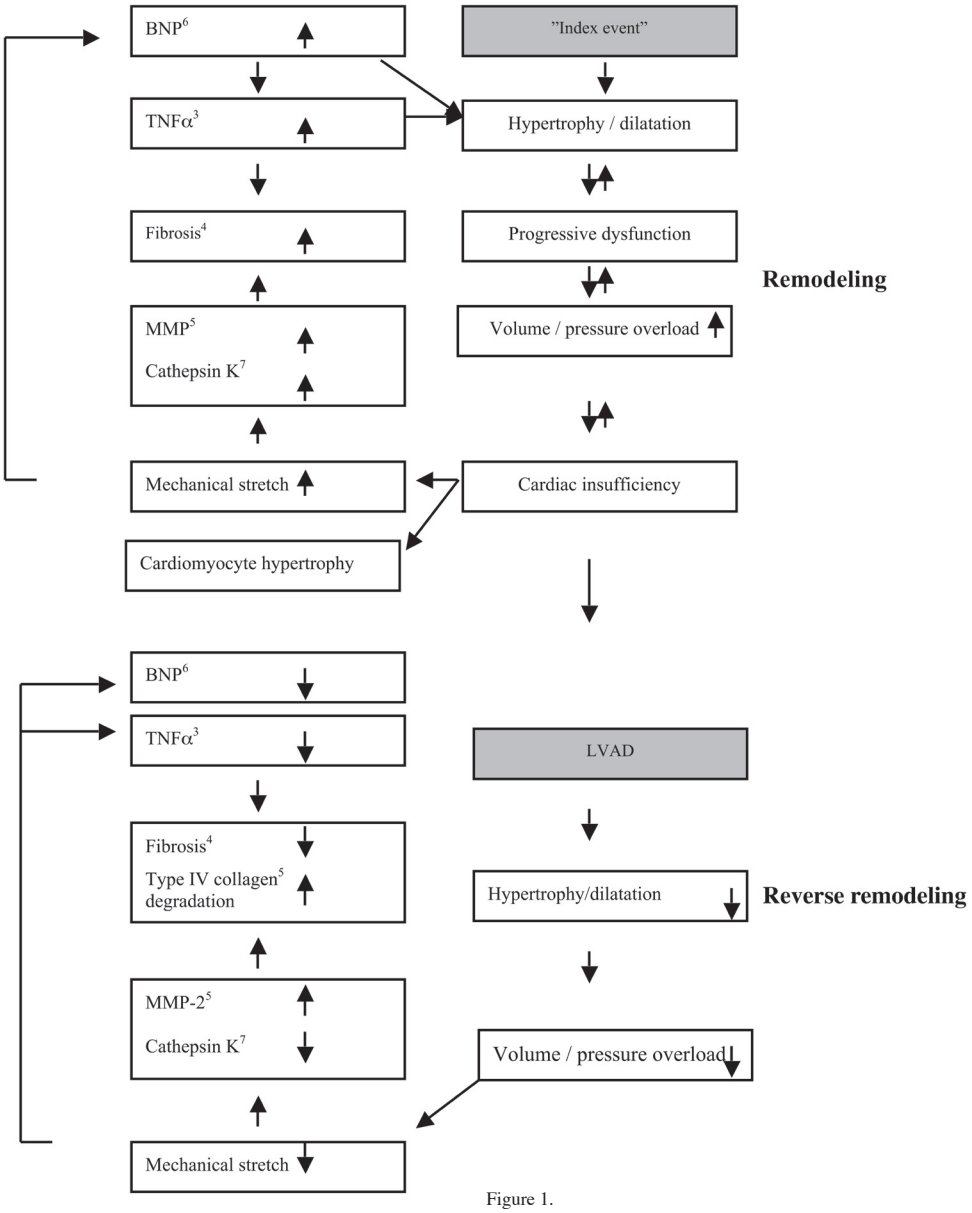

Figure 1.
Schematic overview of the cardiological processes relevant to the subject of this thesis, Alongside the accompanying cellular and molecular events. Nrs. reference to the chapter where these results are observed.

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9

Summary

This thesis describes a study of the changes in the extracellular matrix (ECM) of the myocardium, in patients with end stage heart failure and during mechanical support (Left Ventricular Assist Device support, LVAD) of the left ventricle. The changes during LVAD support may lead to recovery of the heart.

Chapter 1 is a general introduction which provides an overview of heart failure, heart transplantation, and the LVAD. The process of remodeling which leads to heart failure, and of reverse remodeling during LVAD support, are described. A summary is given of the ECM components, and of the role of enzymes, hormones, and cytokines in the myocardial ECM changes in patients before and after LVAD support.

Chapter 2 gives an overview of the role of different cytokine polymorphisms in transplant rejection. Cytokines influence each other's function and production. Single nucleotide polymorphism (SNP) in the genes of cytokines may influence the production level. These SNPs have been studied extensively in the context of acute rejection after organ transplantation. A variety of results have been obtained by many different groups. The main focus in most studies has been SNP genotype of the recipient. However, it is becoming increasingly clear that, in addition to the recipient's immune system, the microenvironment of the donor organ does also contribute to cytokine production, and may thereby influence rejection events.

Chapter 3 concerns a study of TNFα promoter polymorphisms, and their possible contribution to the severity of heart failure and transplant rejection. TNFα plasma levels were measured before heart transplantation, in patients on medication, and in patients supported by a LVAD. TNFα plasma levels were high in patients with end stage heart failure on pharmaco-therapy, compared to healthy controls, but were increased in patients which required LVAD support before heart transplantation. This increase seems to correlate with the G allele at position -308. After LVAD support the TNFα levels were decreased. The A allele at position -308 (in which A is associated with relatively high levels of TNFα) does not correlate with the severity of heart failure and transplant rejection. However, patients having donor hearts with the A-308 polymorphism suffered more rejection episodes than patients receiving hearts with the G allele.

Chapter 4 deals with the changes in the fibrillar collagen network in the ECM. The cardiomyocyte size appeared to decreases by 36% in the first 100 days after LVAD support. This was paralleled by an increase in ECM volume. However, the ECM volume decreased after prolonged LVAD support (>365 days). In patients with end-stage heart failure, the degradation of the fibrillar collagens was high before LVAD support, and their synthesis was low compared to the healthy controls. After LVAD support the opposite was observed: increased synthesis of the fibrillar collagens and decreased degradation. Furthermore, we showed that the quality of the fibrillar collagens increases, because more un-denatured (uncoiled) collagen was detected. These results suggest that after LVAD support the heart becomes smaller and the fibrillar collagen network in the ECM improves.

Chapter 5 describes the changes in the basement membrane (BM) surrounding the cardiomyocytes. Using quantitative PCR (Q-PCR), no changes were observed in terms of collagen α-chain mRNA expression. However, immunohistochemistry did disclose a decreased staining pattern of type IV collagen after LVAD support. Using electron microscopy we showed that in healthy control hearts, and in patients with severe end-stage heart failure, the BM is thick and irregular, in contrast to patients after LVAD support. There were close connections between the ECM collagen fibers and the BM. Post-LVAD the BM became more regular and the lamina lucida and lamina densa were compacter and could be clearly discerned then in the healthy control. However, the connection between the BM and the collagen fibers in the ECM appeared to be disturbed. Furthermore, it was investigated whether matrix metalloproteinases (MMP) plays a role in degradation of the BM. MMP-2, which is able to degrade type IV collagen, was high in patients before LVAD support, but was increased even more in patients on LVAD support. Using gel zymography, active MMP expression was increased in these patients and was localized in the BM. This suggests that MMP-2 degrades type IV collagen in the BM during LVAD support.

Chapter 6 focuses on the role of neuro-hormone brain natriuretic peptide (BNP) in the remodeling process. BNP is a member of the natriuretic peptide family and is mainly synthesized and secreted in the left cardiac ventricle. BNP is known to have natriuretic, diuretic and vasorelaxant properties,and have antagonistic effects on the renin-angiotensin-aldosteron system; it is increased in patients with end-stage heart failure. Measurement of BNP plasma levels is of prognostic value for the assessment of cardiac morbidity and mortality. We did observe an increase of BNP in patients with end-stage heart failure. After LVAD, the BNP plasma levels were decreased. Immuno-histochemical staining indicated that, apart from cardiomyocytes, infiltrating T-cells are also capable of producing BNP.

Chapter 7 describes the role of cathepsin K in the remodelings process. Cathepsin K mRNA and protein expression were high in patients with end stage heart failure before LVAD support. However, after LVAD support the mRNA expression was increased, but the protein level decreased. Immunohistochemistry disclosed that the macrophages in the heart are the main producers of cathepsin K. After LVAD support, their number was decreased. These results indicate that cathepsin K plays an important role in the remodeling process in patients before LVAD support.

Chapter 8 is a general discussion of the investigations included in this thesis.

Currently, LVADs serve as bridge to heart transplantation and, in some patients, even as bridge to recovery. However, recovery of the heart during LAVD support is generally neither complete nor permanent. In this thesis it is demonstrated that the cardiomyocytes, the ECM volume, and the fibrillar collagen network improves in quality and even almost normalizes. Yet, degradation of the basement membrane and of the connections between the collagens in the BM and the cardiomyocytes did also occur. Such connections are very important for the transmission of force between the cardiomyocytes, and as long as they are not restored, LVAD as a temporary tool for recovery would not appear to be a realistic option.

10

Appendix

Samenvatting

Dit proefschrift beschrijft een onderzoek over de veranderingen in de extracellulaire matrix (ECM) van het myocard, bij patiënten met eind stadium hartfalen en gedurende mechanische ondersteuning (LVAD) van de linker kamer. De veranderingen gedurende LVAD ondersteuning kunnen mogelijk leiden tot herstel van het hart.

Hoofdstuk 1 is een algemene introductie die een overzicht geeft van hartfalen, hart transplantatie, en de LVAD. Het proces van remodeling dat leidt tot hart falen, en dat van reverse remodeling gedurende LVAD ondersteuning, worden beschreven. Verder wordt een samenvatting gegeven van de ECM componenten, en van de rol van enzymen, hormonen en cytokinen in de myocardiale ECM veranderingen bij patiënten vóór en nà de LVAD ondersteuning.

Hoofdstuk 2 geeft een overzicht van de rol van verschillende cytokine polymorfismen bij transplantaatafstoting. Cytokinen beïnvloeden elkaars functie en productie. Ook nucleotide polymorfisme (SNP) in de genen van cytokinen kunnen het productieniveau beïnvloeden. Deze SNP zijn uitgebreid bestudeerd in de context van acute afstoting na orgaantransplantatie. Een veelheid van resultaten is verkregen door vele onderzoeksgroepen. In de meeste studies heeft men zich geconcentreed op het SNP genotype van de ontvanger. Het wordt echter steeds duidelijker dat, naast het immune systeem van de ontvanger, ook het micromilieu van het donororgaan tot cytokine productie bijdraagt, en daardoor de afstotingsprocessen kan beïnvloeden.

Hoofdstuk 3 omvat een studie van TNFα promoter polymorfismen, en hun mogelijke bijdrage aan de ernst van hart falen en transplantaat afstoting. TNFα plasma niveaus werden gemeten voorafgaande aan harttransplantatie, bij patiënten die alleen medicatie kregen, en bij patiënten met een LVAD. TNFα plasma niveaus waren hoog in patiënten met eindstadium hartfalen ten opzichte van de gezonde controles. In patiënten met LVAD ondersteuning was dit niveau zelfs nog hoger dan in de patiënten die alleen medicatie kregen. Er lijkt een correlatie te zijn tussen de TNFα plasma levels en het G allel op positie -308. Na LVAD ondersteuning waren de TNFα levels weer genormaliseerd. Er is geen correlatie gevonden tussen het A allel op positie -308 (waarbij A is geassocieerd met relatief hoge TNFα niveau's) met de ernst van het hartfalen en transplantatie afstoting. Ontvangers van donor harten met het positie -308 A allel , hadden wél vaker transplantaat afstoting dan patiënten die een G allel hart ontvingen.

Hoofdstuk 4 beschrijft de veranderingen in het fibrillair collageen netwerk in de ECM. De cardiomyocyte grootte bleek met 36% af te nemen tijdens de eerste 100 dagen na LVAD ondersteuning. Dit ging gepaard met een toename in ECM volume, hoewel na langdurige LVAD ondersteuning (> 365 dagen) de ECM wel in volume afnam. Bij patiënten met eindstadium hartfalen, voor LVAD support, werd een toegenomen afbraak en een verminderde aanmaak van collagenen gevonden ten opzichte van de gezonde controle. Na LVAD

ondersteuning werd het tegenovergestelde gevonden: Verhoogde aanmaak van de collagenen en een verminderde afbraak. Verder toonden we aan dat de kwaliteit van het collagenen netwerk verbeterde, omdat er minder afbraak produkten werden gevonden. Deze resultaten geven aan dat het hart kleiner wordt en het collagenen netwerk in de ECM zich herstelt door LVAD ondersteuning.

Hoofdstuk 5 concentreert zich op de veranderingen in het basaal membraan (BM) rondom de cardiomyocyten. Met behulp van kwantitatieve PCR (Q-PCR) werden geen veranderingen gezien in de mRNA expressie van de collageen α-ketens. Hoewel, met antistoffen tegen type IV collageen vonden we een verminderde type IV collageen aankleuring na LVAD ondersteuning. Elektronenmicroscopische analyse wees uit dat de BM om de cardiomyocyten in gezonde controle harten en in patiënten met eindstadium hartfalen dik en oneffen was, in tegenstelling tot situatie bij de patiënten na LVAD ondersteuning. Verder waren er juist bij normale harten, én voorafgaande aan de LVAD ondersteuning, duidelijke verbindingen tussen de cardiomyocyten en de ECM aanwezig, deze verbindingen werden nog maar sporadisch waargenomen na LVAD ondersteuning.Verder is onderzocht in hoeverre matrix metalloproteinases (MMPs) een belangrijke rol spelen bij de afbraak van de BM. MMP-2, wat in staat is om type IV collageen af te breken, was toegenomen in patiënten voorafgaande aan LVAD ondersteuning, maar nog hoger na LVAD. Dit werd aangetoond via mRNA, immunohistochemie, en gelatine zymografie. Actief MMP bleek aanwezig in het BM. Dit suggereert dat MMP-2 type IV collageen in de basaal membraan afbreekt gedurende LVAD ondersteuning.

In Hoofdstuk 6 wordt het onderzoek beschreven naar de rol van het neuro-hormoon Brain Natriuretic peptide (BNP) bij het remodelings proces. BNP behoort tot de familie van de natriuretische peptiden en wordt voornamelijk gemaakt in de linker ventrikel. BNP heeft natriuretische, diuretische en vaatverwijdende eigenschappen en kan antagonistisch werken binnen het renine-angiotensine-alldosteron systeem; het is verhoogd bij patiënten met hartfalen. Plasma BNP bepaling heeft prognostische waarde bij het vaststellen van cardiale morbiditeit en mortaliteit. In onze patiënten groep werden ook verhoogde BNP plasma waarden gevonden geassociieerd met het terminaal hartfalen. Na LVAD waren de plasma BNP niveau's weer lager. Immunohistochemisch onderzoek wees uit dat BNP tot expressie komt in de hartspiercellen, maar ook in infiltrerende T-cellen.

Hoofdstuk 7 beschrijft de rol van cathepsine K in het remodelings proces. Cathepsine K mRNA en eiwit-expressie was hoog in de patiënten voorafgaande aan LVAD ondersteuning. Na LVAD ondersteuning daalde de mRNA expressie, maar nam de eiwit expressie juist toe. Immunohistochemisch onderzoek wees uit dat de macrofagen in het hart de voornaamste catheprine K producenten zijn. Na LVAD ondersteuning nam het aantal macrofagen af. Het lijkt er op dat cathepsine K een belangrijke rol speelt bij het remodelings proces bij patiënten met eind stadium hartfalen vóór LVAD ondersteuning.

Hoofdstuk 8 is een algemene discussie van het onderzoek beschreven in dit proefschrift. Op dit moment worden de LVADs gebruikt als overbrugging naar harttransplantatie en, in enkele

patiënten, zelfs als brug naar herstel. Het herstel van het hart doorgaans noch compleet, noch permanent. In dit proefschrift is aangetoond dat de cardiomyocyten, de ECM en het collagene netwerk zich herstellen en zelfs lijken te normaliseren. Verder bleek de basaal membraan afgebroken te worden en er werd een afname gezien van de verbindingen tussen de hartspiercellen en de ECM, na LVAD ondersteuning. Deze verbindingen zijn van essentieel belang voor de kracht overdracht tussen de cardiomyocyten, en zolang die verbindingen niet hersteld zijn, lijkt het gebruik van een LVAD als brug naar herstel nog geen reële optie.

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Young investigators Award for Clinical Science, given by the European Society of Cardiology, Lisbon 2005

Nomination for the Caves award 2005, given by the international society for Heart and Lung Transplantation, Philadelphia 2005.

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Het duurt altijd langer dan je denkt,
ook als je denkt
het zal wel langer duren dan ik denk
dan duurt het toch nog langer
dan je denkt.

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Curriculum vitae

Annette Bruggink werd geboren op 17 juni 1970 te Zaandam. In 1986 haalde zij haar MAVO diploma aan de Buitenlandpoort in Vianen. Aansluitend begon zij met haar opleiding tot klinische chemisch analist aan het Versfelt-Ghijssen instituut (MLO) in Utrecht, die zij in 1991 afsloot met een diploma. In februari van dat jaar begon zij met het Hoger Laboratorium Onderwijs (HLO) aan de hoge school van Utrecht in Utrecht. Tijdens deze studie werd een onderzoeksstage gedaan op de afdeling pathologie van het UMC Utrecht onder begeleiding van dr. C.J. Visser en dr. R.A. de Weger. Na het behalen van haar diploma in januari 1994 heeft de schrijfster gewerkt bij Novartis te Basel (Zwitserland) onder dr. H.J. Schuurman aan het mechanisme van xeno transplantatie. Vanaf augustus 1996 werkte zij op de afdeling pathologie van het UMC Utrecht als analist van het HTx team. In 2001 is zij begonnen aan haar promotie onderzoek, eerst op het gebied van cytokine polymorfismen en transplantaat afstoting en later heeft zij het onderzoek van dr. N de Jonge voortgezet op het gebied van mechanische ondersteuning bij patiënten met eindstadium hartfalen. De begeleiding was in handen van prof. dr. J. van den Tweel, dr. R.A. de Weger en dr. F.H.J. Gmelig-Meyling. De resultaten van dit onderzoek zijn beschreven in dit proefschrift. Sinds april 2005 werkt zij als coördinator van de UMCU Biobank. Annette is getrouwd met Theodoor Gijsbers, samen kregen zij een dochter Esther (2001) en dochter Manouk (2002).