

**HUMAN COMPLEMENT REGULATORY PROTEINS IN
HYPERACUTE REJECTION OF CARDIAC XENOGRAFTS**

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**HUMAN COMPLEMENT REGULATORY PROTEINS IN
HYPERACUTE REJECTION OF CARDIAC XENOGRAFTS**

**Humane complement regulerende eiwitten in
hyperacute afstoting van cardiale xenotransplantaten**

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Overige leden: Prof. dr. W. Weimar
Prof. dr. F.G. Grosveld
Prof. dr. F. Claas

Copromotoren: Dr. J.N.M. IJzermans
Dr. R.L. Marquet

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To my parents

To Bart

To Edward

CHAPTER 1

GENERAL INTRODUCTION

1.1 Background and brief history of xenotransplantation

Donor organ shortage can be seen as a consequence of the success of allotransplantation and is mainly a problem of the western countries. Due to the progress in transplantation medicine, more patients with end-stage organ failure can be treated. However, many patients will never receive an organ, since the waiting lists increase every year. According to the United Network for Organ Sharing (UNOS) over 160,000 patients worldwide are registered as candidates for organ transplantation on a waiting list. In the USA more than 79,000 people are awaiting an organ transplant and approximately 16 will die each day without receiving one.

Within the Eurotransplant area (Austria, Belgium, Germany, Luxembourg and The Netherlands) in 1999 3,075 patients received an unrelated cadaveric kidney transplant, whereas 12,393 patients remained on the waiting list. Alternatives for kidney patients are hemodialysis and living-(related) kidney donation. 708 patients received a heart transplant in 1999, and 608 were still on the waiting list at the end of that year. An alternative for heart patients is the artificial heart, but the possibilities of this device are limited. In the same year, 236 lung transplantations were performed and 345 people were waiting for a transplant, whereas 46 patients who needed a heart and lung transplant were waiting for these organs and 28 were transplanted. Up to 25% of patients on the waiting list are dying as no suitable organ is available in time (1). The shortage of human organs has increased interest in xenotransplantation as a possibility to solve this problem.

The first reports of transplantation of animal tissue to humans occurred long before clinical allotransplantation had been established (2). In 1905 slices of rabbit kidney were used to treat renal failure (3). In 1923, a patient lived for 9 days with a grafted lamb kidney (4). Since the 1960's pioneers in the field of xenotransplantation have performed clinical transplantations from primates-to-humans with different success. In table 1 a brief overview is given. In 1964, Reemtsma obtained great success in a series of kidney transplants from chimpanzees to humans with a graft survival of 9 months (5) and Starzl achieved graft survival of 2 months in patients who received baboon kidneys (6). In 1984 Bailey performed an orthotopic transplantation of a baboon heart that functioned for 20 days in a neonate and in 1985 Bailey transplanted a baboon heart, which survived for 1 month (7). In 1992, a pig liver survived for 3 hours in a human (8), and more recently 1996 a pig heart was transplanted into a human but this xenograft survived less than 24 hours (9). Most efforts in xenotransplantation failed because of rejection responses in the recipients (2). In xenotransplantation, i.e. the transplantation of cells, tissues or organs from one species to another different species, e.g. pig-to-human, the intensity of the xenograft rejection depends on the phylogenetical distance

between the donor and the recipient. Based on the different patterns of rejection, this distinction is called concordant and discordant (10).

Table 1. Overview of xenotransplantations from primate-to-human

Donor	Organ	Surgeon	Year	Number	Best result
<i>Chimpanzee</i>	Kidney	Reemtsma	1964	12	9 months
	Liver	Starzl	1964	3	14 days
	Heart	Barnard	1977	1	4 days
<i>Baboon</i>	Kidney	Hitchcock	1964	1	5 days
	Kidney	Starzl	1964	6	2 months
	Heart	Barnard	1977	1	4 days
	Heart	Bailey	1985	1	1 month
	Liver	Starzl	1991	2	2.5 months
<i>Pig</i>	Heart	Religa	1991	1	<24 hours
	Heart	Baruah	1996	1	<24 hours

1.2 Xenotransplantation: concordant / discordant

Concordant xenografts are cells, tissues or organs transplanted in phylogenetically closely related species, i.e. mouse-to-rat, hamster-to-rat, goat-to-sheep, monkey-to-baboon, nonhuman primate-to-human. These grafts reject more vigorously than allografts. The mechanism of rejection is thought to be mediated by a combination of humoral and cellular factors (11). Long-term graft survival can not be guaranteed by conventional immunosuppressive therapy because of the potent induced antibody response in concordant xenotransplantation. However, with immunosuppressive therapy, the reported survival of concordant cardiac grafts in nonhuman primates exceeds 500 days (11;12).

Discordant xenografts are cells, tissues or organs transplanted between phylogenetically widely distinctive species, i.e. guinea pig-to-rat, pig-to-monkey, pig-to-human. Discordant xenotransplant rejection is characterized by the occurrence of hyperacute rejection (HAR) (11;13-15).

1.3 Animals for organ donation

Due to the shortage of allografts for transplantation of patients with end-stage organ failure and by lack of success in developing artificial organs, investigators have been looking for a suitable donor animal (16). The general idea was to generate donor animals transgenic for human complement regulatory proteins. The requirements for an appropriate donor animal are: (1) the size of the donor organs must be conform the size of human organs, (2) the animals must be bred easily, i.e. frequent litters with many descendants, and (3) virological abnormalities must be detectable.

The monkey

The monkey as a potential donor source would be an obvious choice because of the small distance to humans, phylogenetically seen. Furthermore, the anatomy corresponds to the sizes of human organs. However, the monkey has become a rare species and hard to breed (one descendant per year). Through this, the choice using the monkey as an animal source for organ donation, is an emotionally sensitive matter. Next to the ethical reasons, physiologic limitations of the transplant and the possibility of transferring infectious agents from the transplant into the host may also be important (17). Considering the mentioned arguments, the monkey was not chosen as animal source for organ donation.

The pig

The pig seems to be the second potential donor organ source, although it would be a discordant situation. Several years ago, researchers thought it would be difficult to assess how the public was going to react to the possibility of using pig organs for human transplantation in the future, although, we consume pigs at large scale. Furthermore, porcine heart valves have been used for years now for the replacement of diseased human heart valves. Moreover, pig organs correspond to the sizes of human organs, they breed easily and frequent, and have large litters. A possible risk for transmission of zoonoses, particularly pig endogenous retroviruses (PoERV), should be born in mind.

Due to physiological compatibilities as well as ethical and infectious considerations, pigs have now emerged as the most likely source of future xenografts (18;19). The introduction of pigs made transgenic for human complement regulatory proteins and new immunosuppressive regimens seem very promising for future clinical experiments. However, strategies to create transgenic donor animals have been tried in pigs with different success (18;20-23). Knockout techniques are not available in pigs yet, but are under development (23). Additional

clarification of infectious risks and patient strategies are remaining obstacles to application in the clinical arena (18).

1.4 Mouse and rat

Preclinical pig studies, as described above, are useful, but expensive. Moreover, during creation of transgenic animals, also non-transgenic animals are bred due to the Mendelian inheritance of alleles. Although this occurs in smaller animals as well, it is less expensive and easier to perform studies in rodents. An advantage of generating transgenic mice and rats is that the gestation takes 21 days, which is faster as compared to pigs, i.e. 110-118 days (24). In addition, once the pigs have been born, they are too small for experimental usage. Thus, it takes six months before the transgenic pig can be used. Transgenic mice and rats can be used three months after conception. The most important motivation for using rodents in our experiments is that the mechanisms of rejection can be more accurately investigated.

Presently, there are three single transgenic pig and mouse lines available, i.e. hCD55, hCD46 and hCD59. In rats, the hCD55 line is available only. Double transgenic pigs (20;25) and mice (26;27) have been generated recently. Considering the arguments and since the efficacy of multiple human CRPs has never been studied properly in pigs, we have decided to study this in rodents.

1.5 Allotransplantation

Cardiac transplantation

One of the historical landmarks in clinical transplantation has certainly been organ transplantation in general, and heart transplantation in particular. The world's first transplantation of a human heart was performed by Barnard in Cape Town, Africa, on December 3rd 1967, with 18 days survival. A second patient received a human heart on January 2nd 1968 and survived for several years (28). Graft survival after heart transplantation using immunosuppression was equivalent to that of cadaveric kidney transplantation. Presently, the patient one year survival rate after heart transplantation is approximately 80%. Over 90% of them achieve a sufficient heart function and more than 70% get back to normal social life (1).

1.6 Rejection phases in allo- and xenotransplantation

Comparable to the immune response in infectious disease, rejection in organ transplantation is mediated by antibody formation, complement activation and cellular immunity.

1.6.1 Allotransplantation

Due to differences in surface-antigens between two human individuals in allotransplantation, a transplanted organ does provoke a rejection response. These surface-antigens are known as 'major histocompatibility complex' (MHC) antigens or human leucocyte antigen (HLA). MHC contains class I-antigens (A-, B- and C-antigens) and class II-antigens (DP-, DQ- and DR-antigens). These antigens play a pivotal role in the normal immune response. MHC class I-antigens regulate the CD8⁺-T-lymphocytes (T_c- and T_s-cells). Class II-antigens control the CD4⁺-lymphocytes (T_h- and T_s-cells). These helper T-cells help immune responses or induce them. T_h-cells can positively influence the maturation response of other T- and B-cells, and also regulate the cytotoxic and suppressive functions of T_c- and T_s-cells. T_h-cells can be divided into T_{h1}- and T_{h2}-cells. T_{h1}-cells produce cytokines, like Il-2 and IFN- γ , whereas T_{h2}-cells produce Il-2, Il-4, Il-5, Il-6 and Il-10. These cytokines positively and negatively influence the immune response. T_{h1}-cells regulate cytotoxicity and local inflammation, i.e. fight against intracellular pathogens (viruses, bacteria, parasites). T_{h2}-cells are effective in stimulating the proliferation and antibody production of B-cells, and therefore protect humans against 'free' appearing micro-organisms. This is the humoral or antibody mediated immunity, i.e. immunity based on antibodies produced by B-lymphocytes present in the blood.

In human organ transplantation a difference in MHC molecules exists between donor and recipient, leading to an immune response of the recipient. To reduce the risk of organ rejection, HLA-matching is performed prior to transplantation (29) (30). In spite of HLA-matching in the allogeneic situation, rejection can still occur.

Hyperacute rejection occurs within minutes to hours after transplantation and is caused by pre-existing antibodies, which react with MHC-antigens on the vascular endothelium. This leads to complement and endothelial cell activation, resulting in local coagulation. Organ dysfunction and graft loss are the main consequences. The introduction of serological crossmatches before transplantation has decreased the incidence of hyperacute rejection immensely, but it is not completely erased due to the presence of complement-dependent and -independent antibodies (29).

Acute rejection can occur within days or weeks up to one year after transplantation. It is mediated by a cellular immune reaction and type IV allergy reaction. Almost every transplant patient goes through one or more rejection periods. Due to improved management of immunosuppressive therapy the graft survival has drastically improved.

Chronic rejection can occur after months to years after transplantation, leading to graft failure. The most important histological feature in a chronically rejected transplanted heart is intimal hyperplasia. The myocardium appears to be no primary target, however, small triangular-shaped fibrosis in the heart muscle are remainders of infarctions as result of vessel occlusions (31).

1.6.2 Xenotransplantation

Concordant rejection

The concordant rejection process, which is the type of rejection between closely related species, is very similar to that in allograft rejection. To study this type of rejection, in which grafts are rejected after a few days, the hamster-to-rat model is often used (32). Just like in allotransplantation, a variety of immunosuppressive regimens, combining both anti-humoral and anti-T-cell immunosuppression, have successfully been used to extend graft survival (33-35). The first cardiac xenotransplantation was performed in 1964 from a chimpanzee to a 68 old man by Hardy at the University of Mississippi (36). One hour after transplantation the patient died because of an inadequate cardiac output due to the small size of the donor heart. In 1985 a baboon-to-human cardiac xenotransplantation in a neonate was performed (7;37-39). The baboon heart survived for 20 days, graft failure was a result of a progressive humoral response, i.e. an antibody-mediated rejection (7). This suggests that the antibody response is stronger than in allografting and that conventional immunosuppression is not sufficient to suppress concordant rejection (33).

Discordant rejection

The discordant rejection processes can be divided into several rejection phases. First, hyperacute rejection (HAR), second delayed xenograft rejection (DXR) or acute vascular rejection (AVR), and third cell-mediated rejection. These phases are not exactly separated, on the contrary they affect each other.

Hyperacute rejection (HAR)

HAR of discordant transplants has been shown to be due to complement activation, either via the alternative or classical, antibody-dependent, pathway, and to activation of coagulatory and inflammatory pathways, leading to rapid xenograft rejection. HAR of a vascularized graft can take place in as little as 10 minutes, or a few hours (40). The histopathology of HAR is characterized by edema, interstitial haemorrhage, platelet thrombi, intravascular thrombosis, ischemic necrosis and severe injury to endothelial cells, accompanied by destruction of the internal organ structures (17;19;40;41). This process resembles the reaction seen after ABO-mismatched allograft transplantation (33).

Depending on the species combination, HAR is either initiated by the rapid deposition of xenoreactive natural antibodies within the graft and the subsequent activation of the classical pathway of complement activation (e.g. pig-to-primate) (42;43), or is mediated by direct activation of host complement through the graft endothelium (e.g. guinea pig-to-rat combination) (44). In addition to the activation of complement, neutrophil adherence, and vasoconstriction all may contribute to the pathogenesis of HAR in various models of xenograft rejection (45-47).

Xenoreactive natural antibodies (XNA) and Gal α 1-3Gal antibodies

XNA are present in the circulation of all immunocompetent individuals without a known history of sensitization (48;49) and are predominantly directed against only one antigen, a saccharide, Gal α 1-3Gal (50;51). Non-primate mammals, including the pig, express Gal α 1-3Gal (52;53), but humans and most primates do not (54). Natural antibodies in a non-sensitized recipient are primarily, if not solely, IgM. Next to IgM, there is evidence that IgG may have a role in HAR in recipients of discordant xenografts (40;55). Binding of the XNA results in initiation of the complement cascade, leading to the formation of the membrane attack complex (MAC), which causes lysis and opsonisation of cell membranes, and induction of the inflammatory response, resulting in graft destruction.

There are several hypotheses about the source of circulating XNA. They may be produced by a distinctive class of B-cells; the B₁-cells (56). Or they are produced as cross-reacting antibodies during infections with environmental pathogenic agents (33).

The importance of Gal α 1-3Gal as the primary barrier to xenotransplantation was demonstrated by experiments in which anti-Gal α 1-3Gal antibodies were specifically depleted from baboons using immunoaffinity columns. Antibody binding to the transplanted organs was limited and HAR did not occur (57). One approach to overcome the antibody-antigen reaction is to develop lines of pigs with low levels of Gal α 1-3Gal antigen expression. The most obvious approach to developing such lines of donors is to 'knock out' the enzyme that synthesizes the critical sugar, α 1,3-galactosyl transferase. Unfortunately, since embryonic stem cells are not available for the pig, this enzyme cannot be knocked out in pigs as it has been in mice (17;58-61).

Complement

In a discordant model a xenograft is rejected hyperacutely within minutes to hours due to binding of natural antibodies to the vascular endothelium of the xenograft, which causes activation of the complement cascade of the recipient.

The complement system is continuously activated. However, the reason that our body is not destructed, is due to the presence of soluble and membrane-bound complement regulatory proteins (CRPs), which de-activate the cascade. If cells, tissues or organs of a different species are transplanted into the human body, complement factors in the serum of the recipient do not correspond with the CRPs on the endothelium of the donor xenograft. Therefore, the donor CRPs can not de-activate the complement activation, resulting in hyperacute rejection of the xenograft. To this end, genetic modification of donor animals has been performed by implanting human genes, encoding for human CRPs.

Acute vascular rejection (AVR) or delayed xenograft rejection (DXR)

In discordant xenotransplantation, the first obstacle to overcome is HAR, which is primarily mediated by binding of antibody and complement, resulting in activation of endothelial cells. HAR can be averted by removing of natural anti- α Gal antibodies through extracorporeal immunoadsorption (EIA), depletion/inhibition or blocking of complement, and/or the use of donor animals transgenic for CRPs (11), leading to a prolongation of graft survival. But still xenografts are rejected by a process designated as the second immunologic barrier, i.e. acute vascular rejection (AVR) or delayed xenograft rejection (DXR), which does occur in the

absence of T cells. The beginning of AVR coincides with a rise in antibody synthesis, mainly IgG antibody (62;63). These induced antibodies initiate AVR by mechanisms that appear to be independent of complement, although complement fractions may play a role (11;47;64). Since this type of rejection occurs over 2-4 days post-transplant, it is often referred to as acute humoral xenograft rejection.

AVR involves type II endothelial cell activation, including up-regulation of pro-inflammatory antigens, induction of leukocyte adhesion molecules (P-/E-selectin, ICAM-1, VCAM-1), cytokine production, leading to chemotaxis of leukocytes (65), intravascular thrombosis, and infiltration of host granulocytes, monocytes/macrophages and natural killer (NK) cells into the graft. Whether monocytes/macrophages and NK cells have major functions in mediating AVR, or whether they migrate into the xenograft as a response to the presence of antibody, remains unclear.

Activation of endothelial cells

After discordant organ transplantation, binding of XNA to endothelial cells (EC) and activation of complement result in EC activation, leading to destruction of the xenograft. Traditional immunosuppressive regimens are ineffective to block EC activation, only extreme immunosuppression can lead to a prolongation of xenograft survival.

Endothelial cells form a physical barrier, through which the blood and its elements can flow and across which gases and fluids can transverse, but they also promote an anticoagulant environment (40). If activation of endothelial cells occurs, the vascular integrity is lost. In addition, a pro-coagulant environment is created, since activated endothelial cells synthesize tissue factor and lose thrombomodulin. Two types of EC activation are known (66). Type I activation includes retraction of ECs from one another, exposing underlying structures, like collagen. Expression on EC of P-selectin and von Willebrand factor (vWF), and secretion of platelet activating factor (PAF) and other inflammatory mediators occur. Type II EC activation includes loss of anti-thrombotic defenses, heparan sulphate and thrombomodulin, leading to thrombosis. Upregulation of the transcription of many genes encoding for adhesion molecules and cytokines will occur (67), resulting in infiltration of the xenograft with monocytes/macrophages, NK cells, and polymorphonuclear neutrophils (PMN).

Cellular immunity

The probably most difficult immunologic barrier to overcome is the cellular immune response, in which it is assumed that similar mechanisms as in allograft rejection are involved.

Leukocytes

When HAR of a xenograft has passed within a few hours after transplantation, polymorphonuclear neutrophils (PMN) appear to be present in the graft. PMN infiltrate the xenograft more rapidly than NK cells and T-cells, because they adhere to the early adhesion molecule P-selectin, which is upregulated instantly after EC activation (68). In contrast, NK cells use the late adhesion molecules ICAM-1, VCAM-1 and possibly E-selectin for their adherence (69;70).

Monocytes / macrophages

Monocytes can be found in early xenograft rejection. They activate xenogeneic EC by direct cellular contact via membrane bound tumor necrosis factor (TNF). This contact leads to up-regulation of E-selectin, IL-8, monocyte chemoattractant protein-1, and plasminogen activator inhibitor type-1, resulting in intravascular coagulation. These cyto- and chemokines stimulate the monocytes in further transendothelial migration, promoting inflammation, thrombosis and organ infarction (41;71).

B- and T-lymphocytes

B-cells have been detected in rejected porcine xenografts, however, their presence remains unclear. Lysis of xenogeneic targets can be inhibited by anti-CD3 or anti-CD8 antibody, and partially inhibited by anti-CD2. T-cell-independent B-cells may play a central role in late xenograft rejection, but this remains controversial (41).

Natural killer cells (NK cells)

In vitro studies suggested that xenogeneic target cells may be susceptible to NK cell-mediated lysis because their MHC class I molecules are not recognized by specific inhibitory receptors (70). Ex vivo cardiac perfusion experiments suggest that the combination of NK cells and XNA may be a major cause of organ injury. However, no definitive proof currently exists that NK cells are obligatory for the progression of cell-mediated xenograft rejection in vascularized discordant xenografts (41;69;72).

1.7 The complement system

1.7.1 Nomenclature of the complement system (30)

The proteins of the classical pathway and the Membrane Attack Complex (MAC) include C1q, C1r, C1s, C4, C2, C3, C5, C6, C7, C8, C9. Most of these proteins are zymogenes, i.e. pro-enzymes, which must be split before activation. Proteins of the alternative pathway are called factors: Factor B, Ba, Bb, D, H, I and P. When a complement component is activated, this is noted with a *.

Complement receptors can be named

1. after their ligand (e.g. C3a-receptor)
2. with the help of the 'Cluster of Differentiation' (CD-) system
3. according to a numerical system for receptors of the most important fragments of C3, complement receptor type 1 up to 4 (CR1 - CR4)

The descriptions for complement receptors are used concurrently, e.g. receptor for C3b = C3b-receptor = CD35 = CR1.

CR1 (= CD35) is a receptor for C3b and iC3b. It is an opsonin-receptor on neutrophils, monocytes and macrophages, which mediate endocytosis or phagocytosis. It is a co-factor in splicing C3b to iC3b (Factor H is more important), and it is a co-factor in splicing iC3b to C3c and C3dg (CR1 is the only co-factor, which protects host cells against complement attack). On erythrocytes or platelets (dependent on the species) CR1 can mediate the pick-up of opsonised immune complexes or bacteria and transport these to cells of the fixated mononuclear phagocyte-system. On B-lymphocytes CR1 in combination with CR2 can act as a receptor, which brings about lymphocyte-activation.

The CR2 (= CD21)-receptor is primarily restricted to mature B-cells and follicular dendritic cells, and is involved in the activation of B-cells (73). It is also the Epstein-Barr-virusreceptor (74).

CR3 (= CD11b) is probably the major adhesion molecule on monocytes and neutrophils, and is involved in phagocytosis, adhesion of neutrophils and cytotoxicity of cells bearing activated complement components, like C3b.

CR4 (= CD11c) is present on lymphocytes, neutrophils, monocytes and macrophages. It binds iC3b and functions in cell adhesion (75).

Complement proteins can be divided into superfamilies, which share structural and functional features. Complement control proteins, like factor H, a plasma-globulin, C4-binding protein (C4-bp), a heptamer plasma-protein, Decay Accelerating Factor (DAF, CD55), Membrane Co-factor Protein (MCP, CD46), Complement receptor type 1 (CR1, CD35) and type 2 (CR2, CD21), are regulators of complement activation. Factor H, C4-bp, DAF, MCP and CR1 all inhibit formation of C3-convertase-enzymes by the classical and alternative pathways, respectively C4b2a* and C3bBb*. Some of these molecules have other, non-identical functions: inhibition of the binding of C2 to C4b, and Factor B to C3b; and Factor I acts as a co-factor, which is responsible for the catabolism of C3b and C4b.

The complement system is part of the inborn immune system, which distinguishes self, i.e. host, from non-self (30;76). The essence of this distinction is the presence on host tissue of complement regulatory molecules and the absence of these molecules on non-self surfaces. These regulatory proteins protect the host against complement activation by avoiding the C3b-binding to the host cells. Non-self antigens, however, do not have these protective molecules, and therefore many C3b-molecules will be attached to their surface, leading to elimination of the non-self antigens.

The complement system contains many proteins, which mediate a cascade-reaction, at which each enzyme catalyses the next step. Activation of both the classical and alternative pathways takes place either on the membrane or in the fluid-phase. The most crucial component is C3, which connects the classical and alternative pathways. Activation of the cascade can proceed via two pathways:

1. an *antibody-dependent* mechanism, composed by the *acquired* immune system (the classical pathway), initiated by the binding of *specific* antibody of the IgM or IgG class to surface antigens
2. a *non-specific* mechanism, initiated by bacteria, viruses or other 'strange' polysaccharides (LPS) and by some antibodies (IgA) (alternative pathway of the inborn immune system), in which *complement deposition* is localised on the surface of target cells

An overview of the complement cascade is given in figure 1.

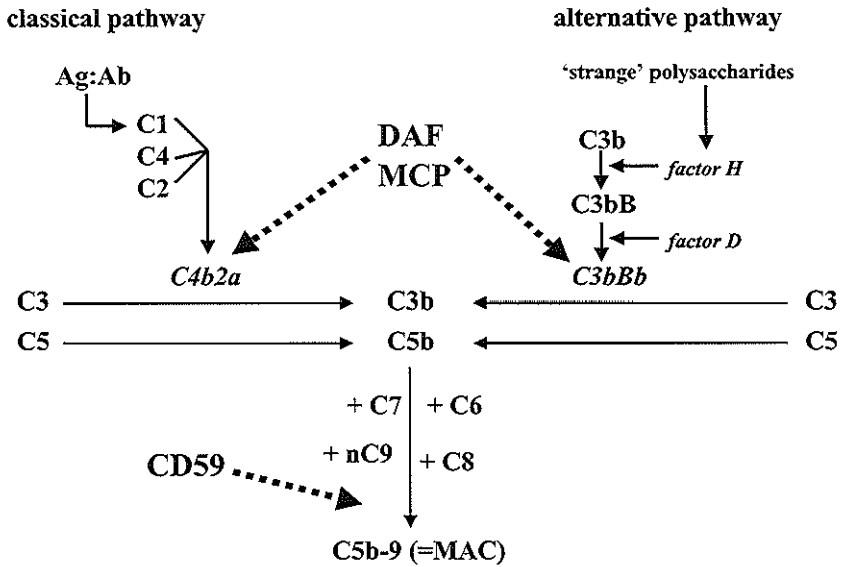


Figure 1. Overview of the complement system.

1.7.2 The classical pathway

This route is mainly activated via the binding of antibodies to antigens, i.e. immune complexes. C1 is the first enzyme complex, which is a pentamolecular Ca^{2+} -dependent complex consisting of one C1q-molecule, two C1r- and two C1s-molecules. When activated C1s splits C4, C4a (a small, mild anaphylatoxic fragment) and C4b* (a large, unstable fragment with an active binding site) appear. Membrane-bound C4b* is a binding site for zymogen C2, resulting in the formation of the classical C3-convertase. This bounded C2 is the substrate for C1s*, which splits off the C2b-fragment. The C2a-fragment stays behind and binds to C4b, forming C4b2a-complex, i.e. C3-convertase of the classical pathway.

C3a and C3b are formed via splitting the C3-molecule with C3-convertase. C3a is an anaphylatoxin. C3b binds with nearby cell surface molecules, like proteins and sugars. Since C3-convertases are localised on non-self surfaces or on immune complexes, C3b-deposition is mainly restricted to these places. Bound C3b acts as a center for further complement activation via the amplification-loop of the alternative route (see below).

Regulation of the classical pathway

In the fluid-phase efficient regulation of the activation of the classical pathway is regulated by two mechanisms. Firstly, a C1-inhibitor binds and inactivates C1r* and C1s*. Secondly, blockade of the formation of C4b2a*, the C3-convertase of the classical route, by Factor I and C4-binding protein (C4-bp). Formation of C4b2a* is inefficient in the fluid-phase because of the presence of Factor I and C4-bp, which together break down C4b. Besides, C4-bp stimulates the dissociation of C2a out of C4b2a*.

Activation of the classical pathway, localised at the membrane, is regulated by inhibition of complementbinding to host cell surfaces via Decay Accelerating Factor (DAF, CD55) and Membrane Cofactor Protein (MCP, CD46).

Anaphylatoxins

C3a, C4a and C5a are anaphylatoxins. They can be described as small complement fragments, which are produced during activation of the complement cascade, and act as mediators in inflammation (77). Anaphylatoxins function via two receptors, the C3a receptor (C3aR), which binds C4a as well, and the C5a receptor (C5aR). C3a and C4a induces smooth muscle contraction, increases vascular permeability and mediates the release of (1) histamine from mast cells, eosinophils, basophils and neutrophils, (b) thromboxane A from macrophages, and (c) lysosomal enzymes from neutrophils. C5a is chemotactic, spasmogenic, increases vascular permeability and mediates the release of pharmacologically active mediators from a number of cell types (78).

1.7.3 The alternative pathway

Normally there is continuous and spontaneous activation of the most crucial component of the cascade, complement factor C3, in the plasma ('tick-over'), resulting in depositions of small numbers of C3b-molecules on host- and non-self-surfaces. On host-surfaces complement regulating molecules mediate the degeneration of C3-deposition and inhibit further complement activation. Activation of the alternative pathway is initiated after 'tick-over'-activation of C3, and C3b-molecules are deposited on non-self surfaces, which lack regulating molecules. The 'tick-over-activation' mediates a continuous production of small C3b*, which has an active binding site for Factor B, resulting in the C3bB-complex. This complex is split by Factor D, through which Factor Ba is released. The residual complex, C3bBb*, is the fluid-phase-C3-convertase of the alternative route. Most of the C3b*, produced by C3-convertase, is hydrolysed and inactivated by water. However, if C3b* would come in contact with non-self surfaces, it will bind and initiate the amplification-loop of the alternative route

(see below). C3b* which will also bind to host surfaces, but due to the presence of CRPs, the amplification-loop will not be initiated.

Regulation of the alternative pathway

Activation of the alternative route in the fluid-phase, when C3b is not membrane-bound, is regulated by proteins, which correspond with complement regulatory molecules, which inhibit the activation of the classical pathway. Factor H stimulates the dissociation of Bb of both C3i and C3b. It also acts as cofactor for Factor I during the catabolism of C3i and C3b.

For the host it is important that the amplification-loop is regulated. This is mediated by a positive feedback-system, which continues until all C3-molecules have been split, unless it will be inhibited by Factor I.

In the membrane-bound phase, DAF and CR1 stimulate the dissociation of C3bBb and release C3b from the complex. Furthermore, CR1 and MCP are co-factors for the splicing of C3b by Factor I (these reactions are analogue with the functions of DAF, MCP and CR1 during the regulation of the activity of the classical-route-enzyme C4b2a*, which is membrane-bound).

Amplification-loop

To stimulate the activated complement system, two amplification mechanisms are known:

1. a 'triggered' enzym cascade. The trigger is the binding of small numbers of C1q-molecules, and the activation of a series zymogenes (pro-enzymes), both resulting in splitting large numbers of C3-molecules
2. a positive feedback-loop, the amplification-loop. The loop starts by splitting small numbers of C3-molecules up to C3b-molecules, resulting in the formation of C3-convertase-enzym, which splits more C3

Host cells possess regulating molecules, which inhibit the amplification-loop by degeneration of C3b up to inactive products. Non-self surfaces have an unregulated amplification, leading to further activation of the complement cascade and destruction of the non-self antigens.

The regulation of membrane-bound C3b is the critical step during the non-specific distinction between self and non-self by the complement system. There are two options for membrane-bound C3b:

1. amplification: C3b plus Factor B \rightarrow convertase-enzyme \rightarrow more deposition of C3b on the same surface
2. inhibition: C3b is degenerated by Factor I, using one of the three co-factors, either Factor H (plasma, fluid-phase) or CR1 and MCP (membrane bound)

The presence of molecules, like DAF, CR1 and MCP effectively limit the formation of C3-convertases on host cells. However, deposition of C3b on non-self surfaces, which do not have the regulating molecules, lead to formation of the alternative-route-C3-convertase and thus more C3b-deposition.

In summary, activation of classical and alternative pathways leads to the formation of C3-convertase, which splices C3 in C3a and C3b. C3-convertase of the classical route is the combination of C4 with C2, resulting in C4b2a*. C3-convertase of the alternative route is the combination of C3 with Factor B, leading to C3bBb*. Both the C3-convertases can be converted in a C5-convertase by adding C3b. C5-convertase catalyses the first step of the cascade, leading to the production of the membrane attack complex (MAC).

1.7.4 Membrane attack complex (MAC)

The final part of the complement cascade contains several complement-molecules, which are circular incorporated into the surface of the target cells. When this circular complex, i.e. the Membrane Attack Complex (MAC), is completed, an irreparable gap appears in the cell membrane. Through this gap leakage of the cell contents take place, which is fatal for the cell.

MAC is formed by enzymatic splicing of C5. Before C5 can be spliced by C5-convertase, it has to be bound to C3b. The classical route-C5-convertase is a trimolecular complex C4b2a3b*, in which C3b is bound to C4b. Alternative-route-C5-convertase is a trimolecular complex C3bBb3b*, in which one C3b is bound to another C3b. When C5 is split, the small peptide fragment C5a is released, which is anaphylatoxic.

Finally, the MAC is formed via non-enzymatic assembly of C5b-C9:

C5b	+ C6	→ C5b6	hydrophilic complex
C5b6	+ C7	→ C5b67	hydrophobic complex → inserts in lipid-bilayer
C5b67	+ C8	→ C5b678	
C5b678	+ 14 C9	→ C5b6789	adding of C9 in steps
C5b6789		= C5b-9	= MAC, gap, hole, plug

Although small lysis occurs after binding of C8 to C5b67, the polymerised C9 causes the main part of the lysis. Hydrophobic molecules, which polymerise to form gaps are involved in general mechanisms against cellular cytotoxicity. T-lymfocytes kill target cells by insertion of a plug, called perforine, into the membranes. Perforine has structural homology with C9. Similar molecules can be found in the granulae of eosinophils. Certain bacterial toxins, like streptolysine O, are gap-forming molecules.

Regulation of the formation of the MAC

Formation of MAC is regulated to reduce reactive lysis, i.e. the hydrophobic C5b67-complexes formed, can insert itself into other cell membranes closely related to the primary surface on which the complement activation is concentrated. Unregulated reactive lysis leads to damage of host cells or tissues. C5b67 can be inactivated in the fluid-phase before it attaches to host membranes by certain proteins, like S-protein (=vitronectine), which is plentiful present in the plasma. It forms the SC5b67-complex, which is unable to insert into lipid-bilayers. Even if C8 or low density lipoprotein (LDL) bind to the SC5b67-complex, no insertion takes place.

Host cells carry membrane proteins, which protect against lysis by the MAC. Protectin (CD59), which is anchored to a glycopospholipidprotein, which is present on lots of cell membranes, and binds to C8 of the C5b-8-complexes. It inhibits the insertion and unfolding of C9 in cell membranes. Furthermore, homologous restriction factor (HRF), also anchored to a glycopospholipid membrane-bound protein, which acts in similar ways as CD59, but is a weaker inhibitor for the insertion of C9.

N.B. Nuclear cells, like the cells of the immune system of the host, are more resistant to lysis by complement than erythrocytes, because they can actively erase the MAC by endocytosis and exocytosis of membrane fragments, which contain MAC.

Consequences of complement activation

Since the complement cascade is the first defence mechanism of the immune system against non-self molecules, sequential activation of the complement system results in:

1. phagocytosis, i.e. opsonisation of target cells, which deposit C3b on their cell membranes
2. lysis, i.e. direct killing of target cells via the formation of the Membrane Attack Complex (MAC), resulting in membrane damage
3. release of peptides active in inflammation, e.g. chemotaxis of leukocytes, basically polymorphonuclear cells (PMN) and macrophages
4. formation of immune complexes
5. induction of specific antibody-responses by enhancing of the localisation of antigens to B-lymphocytes and antigen presenting cells

The process of complement activation constantly takes place in the body and it would destruct all of our cells. Fortunately, a regulatory system is present, which prevents the MAC to be completed and thus averts lysis of cells. This regulatory system comprises the complement regulatory proteins (CRPs), which provide the complement molecules to form the MAC. In humans three inhibitory molecules of the complement cascade have been identified: Decay Accelerating Factor, Membrane Co-factor Protein, both inhibitors at the level of C3 and C5 convertase, and Protectin, an inhibitor at the level of C9. These complement regulatory proteins (CRPs) are present on the endothelium of all organs and they mediate the deactivation of the complement cascade.

1.8 The complement regulatory proteins

As described above, CD46 and CD55 inhibit at the beginning of the complement cascade, at the C3 / C5 convertase level, while CD59 inhibits at the end of the cascade, at the level of formation of the membrane attack complex (MAC).

Decay Accelerating Factor (DAF / CD55)

CD55 is a 70 kDa membrane protein, which is anchored to the membrane by covalent linkage to glyco-phosphatidyl-inositol (GPI). GPI provides fast movement on the membrane layer, and CD55 has the ability to release from and reinsert itself in the membrane via its GPI anchor (67). CD55 is found on virtually every cell and tissue (except NK cells), predominantly on endothelial and epithelial surfaces and also in plasma, urine, tears, saliva, synovial and cerebrospinal fluid (79;80). The structure of the gene for CD55 is comparable to that of CD46. Moreover, DAF and MCP synergize the actions of each other in preventing C3b deposition on the cell surface (81;82).

CD55 enhances the degradation of C3-convertase (= C4b2a) of the classical pathway by (1) inhibition of the binding of C2 to C4b (CD55 of CR1), and by (2) stimulating the dissociation of C2a from C4b (CD55 of CR1). In figure 2 a schematic diagram of the DAF molecule is given.

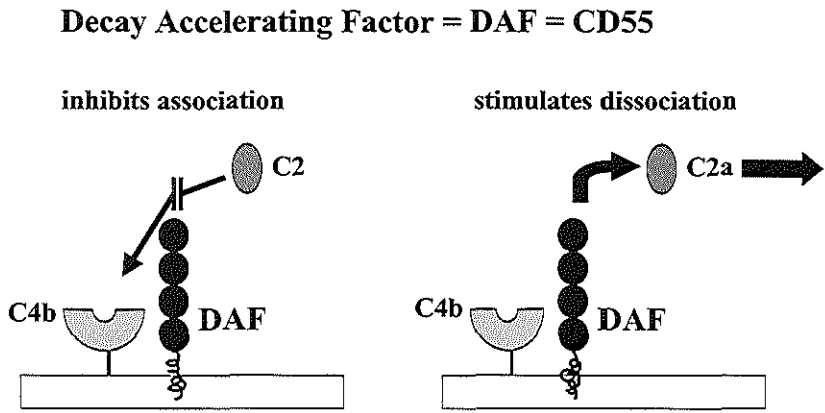


Figure 2. Schematic diagram of CD55 and its function.

Membrane Co-factor Protein (MCP / CD46)

CD46 appears to be in two broad forms of 59-68 kDa and of 51-58 kDa, but there is inherited variability in the quantity of the two forms due to two different alleles of the CD46 gene as is shown by restriction fragment length polymorphism (67;83;84). CD46 is very widely distributed on epithelial and endothelial cells, fibroblasts, platelets, lymphocytes, granulocytes, placental tissue and sperm, hemopoietic cells and peripheral blood cells (except erythrocytes) (84). CD46 is a transmembrane protein, which acts as a co-factor during (1) the cutting of C3b molecules, and (2) the stimulation of the catabolism of C4b by Factor I (MCP of CR1). In figure 3 the MCP molecule and its function is given.

Membrane Cofactor Protein = MCP = CD46

stimulates catabolism of C4b via Factor I

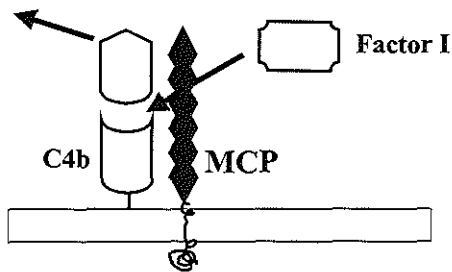


Figure 3. Schematic diagram of CD46 and its function.

Protectin (CD59)

CD59 is a 20 kDa membrane-protein, which is, like CD55, GPI-linked, but shares no other similarity with CD55. The distribution of CD59 over the tissue is as broad as that of CD46 and CD55, although absent from several cell types. It is present in endothelial and epithelial surfaces of most organs, on leukocytes, platelets and erythrocytes, on spermatozoa, placental tissue, thyroid cells, and astrocytes. Like CD55, it is found in urine, saliva, cerebrospinal fluid, breast milk and amniotic fluid with all the GPI anchor intact, including the fatty acid component, and it is not clear whether this is due to the characteristics of the GPI anchor or whether the soluble forms are shed from the cells by some other means (85). Little or no expression is found on many B-cell lines, islets of Langerhans and acinar pancreatic cells, on the proximal tubules of the kidney, hepatocytes, or Kupffer cells (67;86).

CD59 binds to C8 of the C5b-8-complexes (=pre-MAC) and inhibits the insertion and unfolding of C9 in cell membranes (figure 4). CD59 blocks 'low' in the complement cascade.

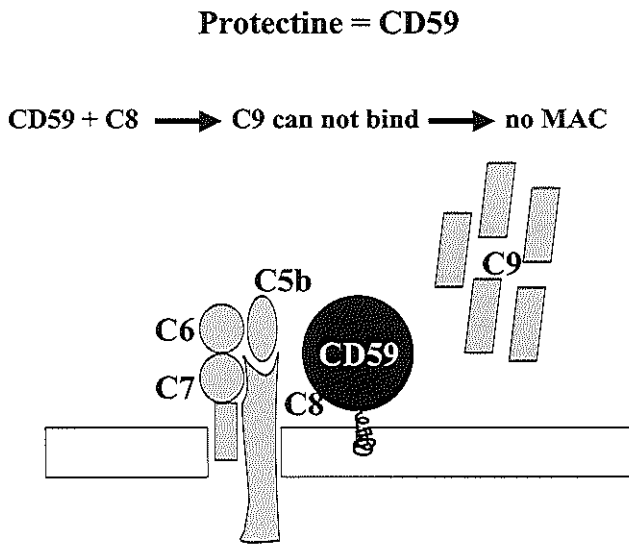


Figure 4. Schematic diagram of CD59 and its function.

1.9 Complement and complement regulatory proteins in human diseases

Complement is implicated in several diseases in humans, both of autoimmune nature and of enhanced vulnerability to systemic infections (67). The mentioned diseases are only examples of the wide spectrum of diseases, in which complement plays a central role.

A clearly described autoimmune disease is Paroxysmal Nocturnal Hemoglobinuria (PNH). The importance of CRPs in the regulation of complement-activity is obvious in PNH, a somatic deficiency of the biosynthesis of glycosylphosphatidylinositol (GPI) anchors in hemopoietic cells. In PNH the synthesis of GPI anchors is blocked. One consequence of this deficiency is that hemopoietic cells show a significant reduction of CD59 and DAF levels on their surface, resulting in an increased sensitivity for complement and spontaneous lysis of erythrocytes. It is a condition characterized in various forms by the absence of CD59, HRF, and/or DAF on red blood cells (87-89). Another unique relation between human disease and complement is the identification of CD46/MCP as a receptor for the measles virus (90). To infect human cells, measles viruses use the complement regulatory protein CD46 as a cell surface receptor (91).

Complement deficiencies are involved in rheumatologic disorders, such as lupus erythematosus (LE) or meningitis (92). An increase in initial and terminal components of the complement cascade results in systemic sclerosis (93). MAC lesions contribute to the development of atherosclerosis and Alzheimer's disease (94). HIV-1 triggers the classical pathway activation in an antibody-independent manner and utilizes the defense mechanism of opsonisation through CR1 and CR3 for enhanced spreading and infectivity (95).

1.10 Modification of the xenogeneic immune response

Vascular xenografts are usually rejected hyperacutely due to naturally occurring antibodies and complement. HAR can be prevented via manipulating either of these systems. 1. Reduction or depletion of anti- α Gal antibodies. 2. Inhibition or depletion of components of the complement system. 3. Genetic engineering of the donor animal.

1. Reduction or depletion of anti- α Gal antibodies can be achieved by plasmapheresis and/or immunoadsorption. However, the continuous removal of the recipient's natural antibodies, is proving difficult, because eventually they return (48).

2a. Human soluble complement receptor 1 (sCR1) inhibits complement activation by acceleration of the splitting of C3/C5-convertases, and for that reason blocking the complement cascade. sCR1 can prolong xenograft survival in the guinea pig-to-rat model from minutes to 32 hours (96).

2b. Depletion of one or more complement components is accomplished by administration of cobra venom factor (CVF), because CVF is an analogue of C3b and therefore activates the complement system continuously (97;98). Complement depletion by CVF can prolong discordant xenograft survival from minutes to days, but complement-independent mechanisms mediate that grafts are finally rejected by AVR (11;99-102).

3. Since continuous depletion of xenogeneic natural occurring antibodies (anti- α Gal) is difficult, specific approaches to therapy, including the genetic engineering of source animals, have been developed. One approach is to create a donor animal, which does not have α Gal, an α Gal knockout. In mice this has already been a success (103). However, it is not yet attainable to develop an α Gal knockout pig, as pig embryonic stem cells are not accessible (23). Another reasonable approach is to generate donor animals transgenic for human complement regulatory proteins, since these proteins inhibit the complement activation. CRPs are mostly species-specific, i.e. CRPs expressed on mouse, rat, porcine tissues do not modify human complement and vice-versa (11). hDAF transgenic pigs have been generated and positive results have been reported. In 1998, the survival of hDAF transgenic pig hearts orthotopically transplanted into baboon recipients was between 18 hours to 9 days (104). However, immunosuppressive therapy was necessary to maintain the graft. Moreover, these hearts were not rejected hyperacutely, but signs of AVR could be detected. In 2000, progress has been made in the discordant pig-to-baboon xenotransplantation model by using a hDAF transgenic pig donor heart and a clinically accepted maintenance immunosuppressive regimen. This combination resulted in the longest survival of an orthotopically transplanted heart ever: 39 days (105). Again inhibition of HAR and subsequent control of humoral and cellular rejection were seen.

1.11 Multiple transgenes

Next to the single transgenic pigs, double transgenic pigs have been bred. Pig hearts double transgenic for both CD59 and CD55 have been generated and transplanted into immunosuppressed baboons. These hearts functioned for 85-130 hours until they were rejected by AVR. Wild-type hearts survived only for 20-80 minutes (20). In Australia

researchers have tried to breed triple transgenic pigs (CD55/CD59/anti- α Gal) and transplanted porcine kidneys to non-immunosuppressed baboons (22). Control kidneys rejected hyperacutely, within 1 hour, but triple transgenic kidneys maintained function for up to 5 days. The triple transgenic kidneys showed cellular infiltration and the recipients developed thrombocytopenia and abnormalities in coagulation within 2 days of transplantation. The findings will have to be studied in rodents, since the development is easier in smaller animals. One double transgenic mouse strain for CD59/CD55 has already been developed and the above results can be confirmed (106). However, it should be kept in mind that these studies have been performed with small numbers of animals.

The aim in the modification of the immune response is to find out which molecules are necessary to avert rejection and maintain graft function. The 'ideal' transgenic donor animal should express the relevant molecules on the vascular endothelium. Once this 'ideal' animal has been generated, cloning would be the best option to maintain the transgene expression.

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CHAPTER 2

AIM OF THE THESIS

1.1 General outline

Heart transplantation is an accepted treatment for certain forms of heart failure. Due to refinement in immunosuppressive therapy, survival rates after heart transplantation are excellent. As a result of this success, more patients are considered for heart transplantation. This development has led to an increased need for donor hearts, while the amount of organ donations remained stable. Up till now, all attempts to substantially increase the number of organs for transplantation purposes have failed. A possible solution for the donor shortage is the use of animal hearts, e.g. pig hearts. However, the rejection process, which occurs after transplantation over a species barrier, i.e. xenotransplantation, is intense and takes place within minutes to hours. Immunosuppressive treatment has hardly any effect, because the rejection process after xenotransplantation is fundamentally different as compared to allotransplantation. In xenotransplantation, hyperacute rejection is caused by (xenoreactive) natural antibodies, leading to activation of the complement system. The activated complement damages the vascular endothelial cells of the donor organ. Normally the complement activities on the endothelium are inhibited by complement regulatory proteins (CRPs). However, during xenotransplantation the regulation of these proteins cannot function properly, because the complement of the recipient is not recognised. Since it is possible to implant human CRPs onto porcine endothelium, the rejection process between pig and human may be less intense and more similar as in allotransplantation.

Research in this field is ongoing. Pigs are bred using genetic manipulation, which carry one or more human CRPs and which may function as donor source for humans. It has not yet come to that, because the process of genetic manipulation is slow. Besides, it has not been demonstrated that the choice of genetic manipulation is the right method. To this end, transgenic mice could be used instead, since human CRPs can be implanted into mice as well. The procedure is less expensive and quicker, since the pregnancy period of mice is only three weeks. These mice are used to study whether the concept of CRP transgenesis is valid. Additionally, the mechanism of rejection can be investigated in rodents, whereas pigs are usually used in the preclinical setting.

1.2 Aims of this thesis

The role of human CRPs in the regulation of hyperacute rejection was studied using

- single transgenic mouse lines. By means of genetic manipulation, mice transgenic for one human CRP, i.e. hDAF, hMCP, hCD59, were produced. Hearts from these mice were perfused with human serum in the Langendorff circuit in order to study whether these hearts were protected against HAR (Chapter 3).

- double transgenic mouse lines. Mouse hearts transgenic for two hCRPs, i.e. hDAF_xhCD59, hDAF_xhMCP, hMCP_xhCD59, were tested whether they would avert HAR to a larger extent than single transgenes by perfusion with human serum in the Langendorff device (Chapter 4).
- a single transgenic rat line. Only one transgenic rat line, hDAF, was available. These rat hearts were analyzed in the Langendorff circuit, either by perfusion with human serum (are the hearts protected against HAR ?) or with human blood (are the hearts protected against cell-mediated rejection as well ?) (Chapter 6 and 7).

Since these isolated heart perfusions were *ex vivo* perfusions, we investigated

- whether hDAF transgenic mouse and rat hearts were also protected against HAR, *in vivo*. Therefore, we transplanted these hearts into primates using a new cannulation-technique (Chapter 5 and 6).

If HAR, which is mediated by antibodies and complement, is averted *in vivo*, an influx of cells, mainly natural killer (NK) cells and macrophages, into the xenograft can occur, resulting in AVR. In Chapter 8, we used the guinea pig-to-rat heart transplantation model to investigate whether AVR could be prevented by specific and non-specific depletion of macrophages and NK cells.

In addition, in xenotransplantation ischemia reperfusion injury may play a pivotal role in graft dysfunction and organ loss. Heme oxygenase-1 (HO-1) is a molecule known to diminish ischemia reperfusion (I/R) injury. Therefore, we investigated whether upregulation of HO-1 would lead to improvement of the performance of mouse hearts perfused with human serum in the Langendorff circuit (Chapter 9).

CHAPTER 3

RELATIVE ROLES OF HCD46 AND HCD55 IN THE REGULATION OF HYPERACUTE REJECTION

C.A.E. VERBAKEL¹, F. BONTHUIS¹, S.E. EERHART¹, M. VAN DIXHOORN²,
F.G. GROSVELD², R.L. MARQUET¹, J.N.M. IJZERMANS¹

¹LABORATORY FOR EXPERIMENTAL SURGERY, ERASMUS UNIVERSITY ROTTERDAM

²LABORATORY FOR CELL BIOLOGY & GENETICS, ERASMUS UNIVERSITY ROTTERDAM

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European Surgical Research (ESSR), 32, Supplement 1, 2000

Introduction

It is now well established that hyperacute rejection (HAR) of discordant xenografts can be overcome by the use of donors that are transgenic for complement regulatory proteins (CRP's). We and others have demonstrated that particularly regulation at the level of C3 leads to the desired effect: mouse hearts transgenic for hCD46 (MCP) showed a marked increased survival time when perfused with human serum, whereas hCD59 transgenes did not (1). The present experiments were done to see whether expression of a second CRP (hCD55) would further improve survival.

Materials & Methods

Animals

Mice

Twelve-to-sixteen weeks old male and female heterozygous transgenic mice, weighing approximately 20 grams, were used. They were generated by microinjection of mouse ova with yeast artificial chromosome (YAC) constructs of the entire genomic DNA encoding for hCD46 or hCD55 at the Department of Cell Biology & Genetics of the Erasmus University (2). The hCD55-mouse strain contained 10 integrated copies in the genome. The hCD46 I-mouse strain contained a single copy of hCD46, whereas the hCD46 II-mouse strain contained 10-12 copies of hCD46. These mice show regulation of mRNA and protein expression as is seen in humans. The expression of the relevant protein on spleen, kidney, heart, liver and lung was verified by anti-hCD55 and anti-hCD46. The background of the hCD55-mice was B₁₀CBA and for hCD46-mice the FVB strain, which were used as controls. B₁₀CBAxFVB mice were used as controls for hCD55xhCD46 mice. hCD55xhCD46 double transgenic mice were bred by crossing the single transgenic mouse strains. The heterozygous F1 offspring was used in this study. Through tailing, each mouse was checked for expression of the single and double transgenes.

The animals were housed in a certified animal breeding facility under standard conditions and had free access to standard mouse chow (AM II, Hope Farms, Woerden, The Netherlands) and water acidified to pH 2.2 ad libitum. The experimental protocols adhered to the rules laid down in the Dutch Animal Experimentation Act (1977) and the published Guidelines on the Protection of Experimental Animals by the Council of the EC (1986). The Committee on Animal Research of Erasmus university Medical Centre, Rotterdam, The Netherlands, had given permission to perform the experimental protocols.

The Langendorff device

To test the effects of complement regulatory proteins, it is necessary to perfuse transgenic mouse or rat hearts with human serum. In figure 1 a schematic overview of the Langendorff circuit is given. Oscar Langendorff deserves credit for first devising a method to permit investigation of the mechanical activity of the completely isolated mammalian heart in 1895 (3).

In our Langendorff system two bottles are present. The large bottle contains Krebs-Henseleit (KH) medium. KH is an artificial medium. If the KH medium is saturated with oxygen, hearts will beat for a long time. The smaller bottle contains the human serum solution, i.e. human serum diluted with KH.

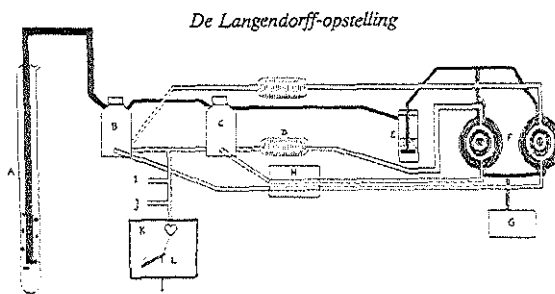


Figure 1. A schematic overview of the Langendorff circuit.

After removal of the heart from the laboratory animal, a cannula is inserted in the ascending aorta. By means of retrograde perfusion, i.e. perfusion against the normal physiological direction, the aortic valves closes (figure 2). Because of this, the medium is driven into the ostia coronaria. After passing through the coronary vascular system, the medium flows off into the coronary sinus, which drains on the right atrium.

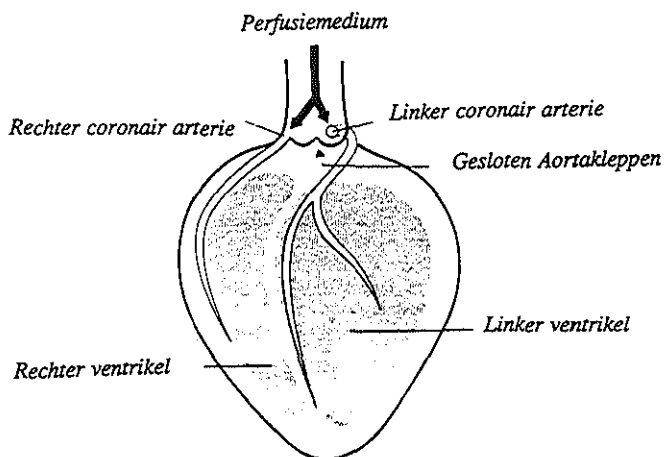


Figure 2. Retrograde perfusion of a Langendorff heart.

When the heart is cannulated, it is connected to the Langendorff system. First, the heart is perfused with KH (bottle 1) to acclimatize. Through the assembly of the medium, the heart regains its contractions again. After switching to human serum solution (bottle 2), the time is registered until complete cessation of heartbeats occurs. The contractility, coronary flow and heart frequency are investigated and restored on a computer. A small fishhook is attached to the apex of the heart, which is connected to a tension-meter. This meter transmits a signal to the computer. Pressure, temperature and humidity are being registered. Oxygen saturation is determined within one minute by means of an auto-analyzer.

Saturation, i.e. providing oxygen, of the medium is realised by means of an artificial lung (figure 3). The 'lung' is a cylinder with an outer chamber, in which oxygen is supplied and removed via two tubes, which are connected to the cylinder. On the inside of the cylinder, long semi-permeable tubing is twisted. Medium coming from the stock-bottle flows through this tubing. The oxygen exchange occurs according to the "tegenstroomprincipe", medium flows through the semi-permeable tubing from right to left; the oxygen in the outer chamber of the cylinder from left to right. The oxygen diffuses from the outer chamber into the medium. Finally, the medium returns to the stock-bottle, where it is heated up to 37°C (4).

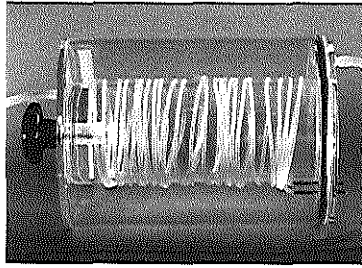


Figure 3. Artificial lung of the Langendorff model.

Surgical procedure

Mice were anaesthetised with iso-flurane inhalation (Pharmachemie B.V. Haarlem, Holland) and the abdominal wall was opened. 0.6 mL Heparin (50 IU/mL) was injected into the abdominal inferior vena cava. Via two lateral incisions the thorax was opened and the heart was removed quickly. A 22-gauge indwelling cannula was inserted into the aorta and secured with a 4.0 ligature. The heart was directly perfused with oxygenated Krebs-Henseleit solution using a syringe and directly linked to the Langendorff device, as described by Verbakel et al. (5). Haemaccel was added to optimize the performance of the mouse hearts during perfusion. Before switching to human serum, acclimatisation of the hearts was accomplished by perfusion with Krebs-Henseleit-Haemaccel solution for 10 minutes. Hearts were monitored constantly for heart rate and flow using multichannel registration. Survival time was defined as the time between the start of serum perfusion and complete cessation of heartbeats.

Human serum

Frozen, pooled, human O-type plasma was obtained from the blood bank of the University Hospital Dijkzigt (Rotterdam, The Netherlands). Human serum was made out of human plasma by adding 0.5 mL Thrombin to 200 mL human plasma, inducing clotting. After removal of the clot, the serum was diluted with Krebs-Henseleit-Haemaccel solution to obtain the perfusion concentration of 15%. For each experiment fresh human serum was prepared.

Immunohistochemistry

After cessation of heartbeats each heart was cut in two. One sample was fixed in 3.6% formaldehyde solution. Following dehydration and paraffin embedding, 4 μm -thick sections were cut and stained with Hematoxylin & Eosin (H&E). The other part of the sample was snap frozen and stored at $-80\text{ }^{\circ}\text{C}$ until sectioned for immunohistochemistry. Frozen tissue sections (7 μm -thick) were prepared in a Microm HM 560 cryostat and stained with rabbit

anti-human C3c Fluorescein-Iso-Thio-Cyanate (FITC). The anti-human C3c-staining was done as described in detail by Verbakel et al. (5). Dilution of the primary antibody was 1:400. Stained sections were examined under a fluorescence microscope. The intensity of C3c-expression on the heart tissues was scored blindly via semi-quantitative analysis: 0 = no staining, 0.5 = mild staining, 1 = dense staining.

Experimental groups

The following mouse lines were tested:

Group	n
Group 1: controls hCD46 I	5
Group 2: hCD46 I	6
Group 3: controls hCD46 II	5
Group 4: hCD46 II	6
Group 5: controls hCD55	12
Group 6: hCD55	11
Group 7: controls hCD55xhCD46	5
Group 8: hCD55xhCD46	7

All hearts were first perfused with Krebs-Henseleit for 10 minutes, before switching to 15% human serum.

Statistical analysis

The non-parametric Mann-Whitney test was used to evaluate the differences (considered significant when $P < .05$) in mean survival time (MST) between groups.

Results

Survival time

In table 1 survival times and mean survival times (in minutes) of the experimental groups are given. Both mouse hearts transgenic for hCD46 I (group 2) and hCD46 II-mouse hearts (group 4) survived significantly longer than their controls (group 1 and 3) ($P < .05$). Moreover, the hCD55-mouse hearts (group 6) beated significantly longer than controls (group 5) as well ($P < .05$). The survival times of hCD55xhCD46-hearts (group 8) and hCD46 I-hearts (group 2) was similar. hCD55xhCD46-hearts did not beated longer than hCD46 II- and hCD55-hearts (group 4 and 6), but they beated three times longer than their controls (group 7).

Table 1. Survival times and mean survival times (MST) of transgenic mouse hearts and their controls (in minutes) perfused with 15% human serum in the Langendorff device

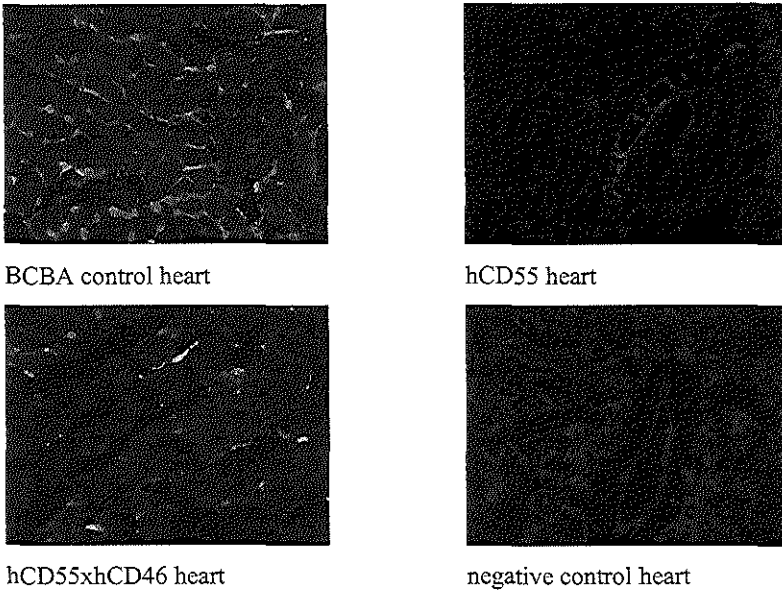
Type	Survival Times	MST
hCD46 I	9-10-10-11->45	>17*
Controls hCD46 I	2-5-5-6-7-7	5.3
hCD46 II	15-22-30-45->50	>32*
Controls hCD46 II	3-4-7-9-14-20	9.5
hCD55	10-15-22-23-29-30-30-30-30-35-40-52	28.8*
Controls hCD55	3-4-4-5-5-6-7-7-8-8-11	6.2
hCD46 II x hCD55	15-16-17-17-20	17*
Controls hCD46 II x hCD55	4-4-5-5-7-7-8	5.7

* $P < .05$

Immunohistochemistry

Immunohistochemical examination of the hCD46IIxhCD55-hearts revealed diffuse staining of C3c in contrast to hCD55- and hCD46-hearts, which showed patchy staining around the vessels, not involving the parenchymal structures (figure 5).

Figure 5. Anti-human C3c-staining of transgenic and non-transgenic mouse hearts perfused with human serum in the Langendorff system. C3c deposition is seen in BCBA controls, whereas hCD55 and hCD55xhCD46 hearts showed reduced C3c-levels.



Conclusions

Transgenesis with one complement regulatory protein, i.e. hCD46, has been demonstrated to be effective to increase survival of xenogeneic organs, and thus that these hearts were protected against HAR provoked by ex vivo perfusion with human serum in the Langendorff device. The aim of the present study was to see whether the xenograft survival would improve, if a second CRP (hCD55) was added.

Our results indicate that hCD46-hearts show a threefold increase in survival as compared to controls, irrespective of the degree of tissue expression (MYII>>>MYI). Furthermore, that complement regulation at the level of C3 by hCD55 leads to similar prolongation as regulation by hCD46. The main conclusion of this experiment is that expression of both hCD46 and hCD55 does not further enhance survival time. It thus appears that, as far as protection against HAR is concerned, inhibition at the level of C3 by one CRP provides the maximal attainable effect. However, it should be kept in mind that in the current experiments heterozygous animals were used; it may be expected that, if we had used homozygous strains, further improvement of xenograft survival can be obtained.

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CHAPTER 4

COMPLEMENT REGULATORY PROTEINS TO PREVENT HYPERACUTE REJECTION: IS MORE BETTER ?

C.A.E. VERBAKEL¹, S. DEKKER², S. VAN DUIKEREN¹,
R.L. MARQUET¹, J.N.M. IJZERMANS¹

¹LABORATORY FOR EXPERIMENTAL SURGERY, ERASMUS UNIVERSITY ROTTERDAM

²LABORATORY FOR CELL BIOLOGY & GENETICS, ERASMUS UNIVERSITY ROTTERDAM

submitted to Xenotransplantation

Abstract

Hyperacute rejection (HAR) of discordant transplants is caused by direct and indirect activation of complement (C). Decay Accelerating Factor (DAF/CD55), Membrane Co-factor Protein (MCP/CD46), both inhibitors at the level of C3, and Protectin (CD59), an inhibitor at the level of C9, are complement regulatory proteins present on the endothelium of all organs. They mediate the de-activation of the complement cascade. We demonstrated earlier that mouse hearts transgenic for human CD55 (hCD55) or human CD46 (hCD46) are protected against HAR, provoked by human serum. The aim of our study was to compare the survival of single and double transgenic mouse hearts in the Langendorff circuit by means of ex vivo heart perfusion with human serum. The survival of mouse hearts transgenic for hCD55 or hCD46 perfused with human serum was significantly longer than non-transgenic controls. On the contrary, hCD59 hearts survived as long as their controls. In the double transgenic groups, hCD59xhCD55 and hCD59xhCD46 hearts survived longer as compared to non-transgenic controls. But compared to single transgenes they did not perform better. Surprisingly, hCD55xhCD46 hearts beat shorter as compared to hCD55 or hCD46 single transgenic mice. In conclusion, (1) hCD46 or hCD55 mouse hearts are protected against HAR, whereas hCD59 hearts are not, (2) hCD59xhCD55 and hCD59xhCD46 double transgenic mouse hearts are better protected against HAR than their controls, but not better than single transgenic mouse hearts (hCD55 or hCD46), (3) the combination hCD55xhCD46 is even worse than hCD55 or hCD46 alone, (4) to prevent HAR it is enough to be transgenic for one complement regulatory protein, which inhibits at the level of C3.

Introduction

In the western countries it is a well-known fact that the number of human donor organs is limited (1-3). Xenotransplantation, ie transplantation of cells, organs or tissues between phylogenetically different species, seems to be a reasonable solution. The success of xenotransplantation depends predominantly on how well the immune response can be controlled. The rejection response starts with hyperacute rejection (HAR), followed by acute vascular rejection (AVR). These rejection phases lead to organ dysfunction and graft loss. HAR of discordant transplants is caused by xenoreactive natural antibodies (XNA) binding to the xenograft, leading to activation of the complement (C) system, either via the alternative or the classical, antibody dependent, pathway (4). The complement system is activated continuously because of the constant 'physiological' invasion of viruses, bacteria, and other polysaccharides. Our own cells are protected against this complement-mediated attack by the presence of complement regulatory proteins on the vascular endothelium of all organs. In xenotransplantation, the general idea is to manipulate the donor animal in order to minimize the incompatibility between the complement regulatory proteins present on the donor endothelium and complement factors in the plasma of the recipient. Therefore, introduction of human Decay Accelerating Factor (DAF/CD55), Membrane Co-factor Protein (MCP/CD46), both inhibitors at the level of C3, and Protectin (CD59), an inhibitor at the level of C9, into the genome of the donor animal can be performed. We demonstrated earlier that mouse hearts transgenic for human CD55 (hCD55) or human CD46 (hCD46) were protected against HAR, whereas hCD59 hearts were not (5-8). The aim of the present study was to test the protective effect against HAR of double transgenic mouse hearts in the Langendorff circuit by means of ex vivo heart perfusion with human serum. By using the F1 offspring of parental mice transgenic either for hCD55, hCD46 or hCD59, we were able to investigate which combination of complement regulatory proteins was the most optimal in protecting against HAR.

Materials and Methods

Animals

Mice

Production of single transgenic mice was accomplished by micro-injecting mouse ova with entire genomic DNA encoding for hCD55, hCD46 or hCD59 using yeast artificial chromosomes (YAC), which has been described by Yannoutsos et al. (9). The hCD55-mouse strain contained 10 integrated copies in the genome. For the hCD46- and hCD59-mouse

strains this was 10-12 and 3 integrated copies, respectively. These mice show regulation of mRNA and protein expression as is seen in humans. The expression of the relevant protein on spleen, kidney, heart, liver and lung was verified by anti-hCD55, anti-hCD46 and anti-hCD59. Via blot analysis the expression of the transgenes was evaluated as described by Yannoutsos et al. (10). The Southern blot technique showed that the YAC DNA of the transgenes was incorporated into the mouse genome. The background of hCD55 and hCD59 mice was B₁₀CBA, and used as control. For hCD46 mice this was the FVB strain. By crossing the single transgenic mouse strains, either transgenic for hCD55, hCD46 or hCD59, male and female heterozygous double transgenic mice were bred. These heterozygous F1 offspring was used in this study. Through tailing, each mouse was checked for expression of the double transgenes. Splenocytes of mice transgenic for hCD55xhCD46, hCD59xhCD55 and hCD59xhCD46 were analyzed by FACS-analysis, and mouse hearts were tested for expression of the transgene(s) by immunohistochemistry.

The animals weighed approximately 20 grams and were twelve-to-sixteen weeks old. The animals were housed in a certified animal breeding facility under standard conditions and had free access to standard mouse chow (AM II, Hope Farms, Woerden, The Netherlands) and water acidified to pH 2.2 ad libitum. The experimental protocols adhered to the rules laid down in the Dutch Animal Experimentation Act (1977) and the published Guidelines on the Protection of Experimental Animals by the Council of the EC (1986). The Committee on Animal Research of Erasmus university Medical Centre, Rotterdam, The Netherlands, had given permission to perform the experimental protocols.

FACS-analysis of splenocytes

Expression of human complement regulatory proteins on 25000 mouse splenocytes of single CD55, single CD46, single CD59 or double CD55/CD46, CD59/CD55, CD59/CD46 transgenic mice was assessed using a FACS scan flow cytometer, as described earlier (11). Mouse monoclonal antihuman-CD55-, -CD46-, and CD59-FITC-antibodies were used.

Verification of the transgene expression

The expression of human complement regulatory proteins was verified by immunohistochemical staining of the double transgenic heart tissues with primary anti-human-CD55-, -CD46- or -CD59-antibodies, which were biotinylated. Avidin-Texas Red conjugate was used as secondary antibody. Hearts were sliced in sections of 7 µm with a cryostat and stored at -20°C. Later these tissue sections were air-dried for 30 minutes, after which they were fixed in acetone for 1 minute at 4°C. After air-drying for 10 minutes, the

sections were incubated with the primary antibody (either biotinylated CD55, CD46 or CD59 antibody; dilution 1:10) for 1 hour. After washing with Phosphate Buffered Saline / Bovine Serum Albumine (PBS/BSA) (3x10 minutes), the sections were incubated with Avidin-Texas Red conjugate (1:500) for 30 minutes. Sections were washed with PBS/BSA (3x2 minutes) again. Finally, the sections were dehydrated using a series of 70%, 90% and 100% ethanol, washed in Xylol, and covered with Entellan.

The Langendorff circuit

Prior to the surgical procedure, mice were anaesthetised with iso-flurane inhalation. The abdominal wall was opened and 0.6 mL Heparin (50 IU/mL) was injected into the abdominal inferior vena cava. Via two lateral incisions the thorax was opened and the heart was removed quickly. A 22-gauge indwelling cannula was inserted into the aorta and secured with a 4.0 ligature. The heart was contiguously perfused with oxygenated Krebs-Henseleit solution using a syringe and directly linked to the Langendorff device, as described by Verbakel et al. (8). Haemaccel was added to optimize the performance of the mouse hearts during perfusion. Before switching to human serum, acclimatisation of the hearts was accomplished by perfusion with Krebs-Henseleit-Haemaccel solution for 10 minutes. Hearts were monitored constantly for heart rate and flow using multichannel registration. Survival time was defined as the time between the start of serum perfusion and complete cessation of heartbeats.

Human serum

Frozen, pooled, human O-type plasma was obtained from the blood bank of the University Hospital Dijkzigt (Rotterdam, The Netherlands). Human serum was made out of human plasma by adding 0.5 mL Thrombin to 200 mL human plasma, inducing clotting. After removal of the clot, the serum was diluted with Krebs-Henseleit-Haemaccel solution to obtain the perfusion concentration of 15%. For each experiment fresh human serum was prepared.

Experimental groups

Group	Krebs-Henseleit	Human serum	n
Group 1: B ₁₀ CBA control hearts	Yes	yes	15
Group 2: FVB control hearts	Yes	yes	13
Group 3: hCD55 hearts	Yes	yes	15
Group 4: hCD46 hearts	Yes	yes	16
Group 5: hCD59 hearts	Yes	yes	21
Group 6: hCD59xhCD55 hearts	Yes	yes	14
Group 7: hCD55xhCD46 hearts	Yes	yes	18
Group 8: hCD59xhCD46 hearts	Yes	yes	5

All hearts were perfused for 10 minutes with Krebs-Henseleit to acclimatize, before switching to 15% human serum.

Immunohistochemistry of 'rejected' hearts

After cessation of heartbeats each heart was cut in two. One sample was fixed in 3.6% formaldehyde solution. Following dehydration and paraffin embedding, 4 μm -thick sections were cut and stained with Hematoxylin & Eosin (H&E). The other part of the sample was snap frozen and stored at $-80\text{ }^{\circ}\text{C}$ until sectioned for immunohistochemistry. Frozen tissue sections (7 μm -thick) were prepared in a Microm HM 560 cryostat and stained with rabbit anti-human C3c complement Fluorescin-Iso-Thio-Cyanate (FITC), rabbit anti-human IgA, IgG and IgM, and rabbit anti-human C9. The anti-human C3c-staining was done as described in detail by Verbakel et al. (8). Anti-human -IgA-, -IgG-, -IgM-staining was done in the same way as antihuman C9 staining as described earlier (8); dilution of all primary antibodies was 1:400.

Anti-human C3c-stained sections were examined under a fluorescence microscope. Anti-human C9-, IgA-, IgG-, IgM-stained sections were scored under a light microscope. The intensity of C3c- and C9-expression on the heart tissues was scored blindly via semi-quantitative analysis: 0 = no staining, 0.5 = mild staining, 1 = dense staining. IgA-, IgG-, IgM-intensities were also analyzed semi-quantitatively: 0 = no staining, 0.5 = mild staining, 1 = moderate staining, 2 = dense staining.

Statistical analysis

The non-parametric Kruskal-Wallis test was used to check which test had to be done. To analyse the differences in survival time and C9-depositions between the experimental groups, the non-parametric Mann-Whitney test was used. The Student's t-test was performed on the results obtained from the anti-human C3c-, IgA-, IgG- and IgM-staining. The level of significance being $P < .05$.

Results

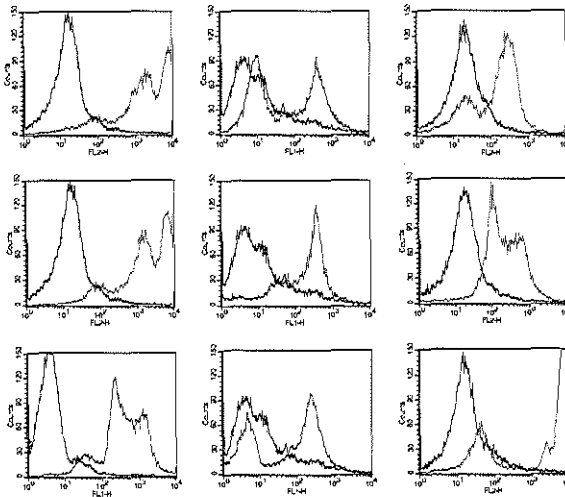
Expression of transgenes

Splenocytes of double transgenic mice showed a strong expression of the transgene by FACS analysis. This is illustrated in the second and third row in figure 1. Verification of the expression of the single transgenic mouse splenocytes also showed a strong expression (the first upper three pictures in figure 1; see legend p. 62).

Immunohistochemical staining

Mouse hearts double transgenic for hCD55xhCD46, hCD59xhCD55 and hCD59xhCD46 exhibited the complement regulatory proteins on both the endothelium and along the cardiomyocytes. In figure 3 the results of these stainings are shown.

Figure 1. FACS-analysis of 25,000 splenocytes of single and double transgenic mice.



Legend figure 1.

hCD59 (red) control (black)	hCD55 (green) control (black)	hCD46 (red) control (black)
hCD59xhCD55 (hCD59: red) control (black)	hCD59xhCD55 (hCD55: green) control (black)	hCD59xhCD46 (hCD46: red) control (black)
hCD59xhCD46 (hCD59: green) control (black)	hCD55xhCD46 (hCD55: green) control (black)	hCD55xhCD46 (hCD46: red) control (black)

Survival time

In table 1 and figure 2 mean survival times (in minutes) of the experimental groups are shown. Mouse hearts transgenic for hCD55 (group 3) or hCD46 (group 4) perfused with 15% human serum beated significantly longer than their non-transgenic controls (group 1 and 2, respectively). On the contrary, hCD59 hearts (group 5) survived as long as their controls (group 1). In the double transgenic groups, hCD59xhCD55 (group 6) and hCD59xhCD46 (group 8) hearts beated longer as compared to non-transgenic controls (group 1 and 2). But in contrast to single transgenes (group 3,4 and 5) they did not beat longer. hCD55xhCD46 (group 7) hearts beated as long as their controls, and shorter as compared to hCD55 or hCD46 single transgenic mice.

Table 1. Survival times and mean survival times (MST) ± SD of mouse hearts perfused with 15% human serum in the Langendorff device (in minutes)

Group	Survival times	MST ± SD
1. BCBA controls	3-4-4-4-4-5-5-5-5-5-6-6-8-8-8	5.3 ± 1.6
2. FVB controls	2-3-3-3-4-4-4-4-5-5-5-6	4.0 ± 1.1
3. hCD55	5-5-5-5-5-6-7-10-10-11-15-15-30-30-30	12.6 ± 9.6*
4. hCD46	3-3-4-6-6-6-10-10-11-13-15-15-15-15-30-30	13.7 ± 8.3*
5. hCD59	2-2-2-3-4-4-4-5-6-8-8-11-14-16-18-30-30-30-30-30-30	13.7 ± 11.5
6. hCD59xhCD55	4-5-5-10-13-15-15-20-20-30-30-30-30-30	18.2 ± 10.3*
7. hCD55xhCD46	2-2-2-2-2-2-3-3-3-3-4-4-5-6-7-12-15-30	5.9 ± 7.0
8. hCD59xhCD46	2-9-13-30-30	16.8 ± 12.7*

* P < .05

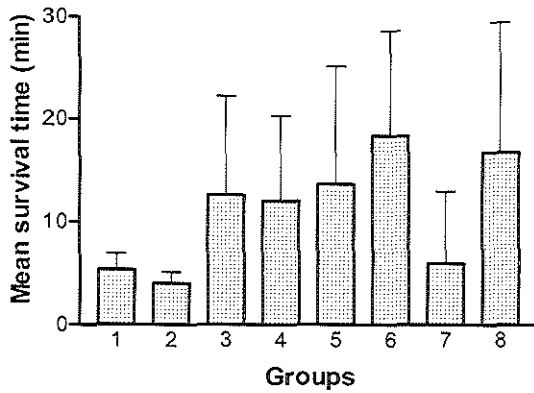


Figure 2. Mean beating times in minutes per group. Group 1: B₁₀CBA controls, group 2: FVB controls, group 3: hCD55, group 4: hCD46, group 5: hCD59, group 6: hCD59xhCD55, group 7: hCD55xhCD46, group 8: hCD59xhCD46. (1vs3 P = .004, 2vs4 P = .001, 1vs6 P = .001, 1vs8 P = .046, 2vs8 P = .045, 3vs7 P = .002, 4vs7 P = .003, 6vs7 P = .0001).

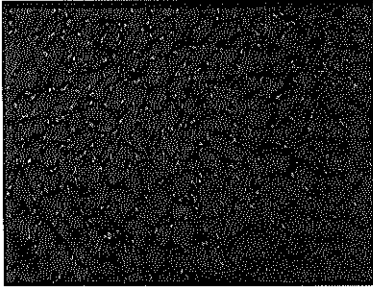
C3c, C9, IgA, IgG, IgM depositions

H&E-stained sections of transgenic and non-transgenic hearts both exhibited edema, but these findings were more prominent in the non-transgenic hearts. In table 2 mean scores of C3c, C9, IgA, IgG, IgM depositions are given. No significant differences were found in depositions of C3c, C9, IgA, IgG and IgM between non-transgenic and transgenic mouse hearts. Deposition of C3c and C9 was mainly localised diffuse along the cardiomyocytes, and sometimes at the vascular endothelium. IgA, IgG and IgM depositions were localised at both the endothelium of large and small blood vessels and diffuse along the cardiomyocytes as well.

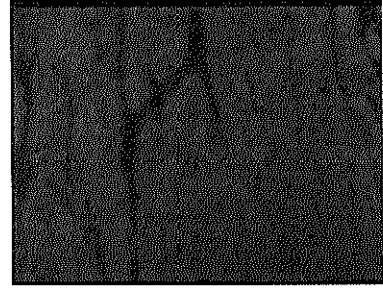
Table 2. Mean semiquantitative scores \pm SD of C3c, C9, IgA, IgG, IgM depositions.

Group	C3c	C9	IgA	IgG	IgM
1. BCBA controls	0.3 \pm 0.3	0.6 \pm 0.2	0.8 \pm 0.4	0.9 \pm 0.5	0.7 \pm 0.3
2. FVB controls	0.2 \pm 0.3	0.5 \pm 0.4	0.7 \pm 0.4	1.0 \pm 0.5	1.0 \pm 0.6
3. hDAF	0.4 \pm 0.4	0.5 \pm 0.3	1.0 \pm 0.7	1.0 \pm 0.5	0.7 \pm 0.3
4. hMCP	0.5 \pm 0.5	0.6 \pm 0.4	0.9 \pm 0.4	0.8 \pm 0.4	0.8 \pm 0.4
5. hCD59	0.3 \pm 0.4	0.5 \pm 0.3	0.8 \pm 0.5	0.8 \pm 0.4	0.8 \pm 0.4
6. hCD59xhDAF	0.4 \pm 0.3	0.5 \pm 0.4	0.6 \pm 0.3	1.0 \pm 0.6	0.7 \pm 0.3
7. hDAFxhMCP	0.3 \pm 0.4	0.5 \pm 0.3	0.8 \pm 0.5	0.8 \pm 0.4	0.7 \pm 0.4
8. hCD59xhMCP	0.5 \pm 0.6	0.5 \pm 0.3	0.6 \pm 0.5	0.6 \pm 0.5	0.6 \pm 0.5

Figure 3. Immunohistochemical staining of single and double transgenic mouse hearts. A. hCD55 mouse heart stained with anti-hCD55, B. negative control, C. hCD59xhCD46 stained for hCD59, D. hCD59xhCD46 heart stained for hCD46. hCD46 and hCD59 single transgenic hearts looked the same as hCD55 (A). This also holds for the other double transgenic hearts.



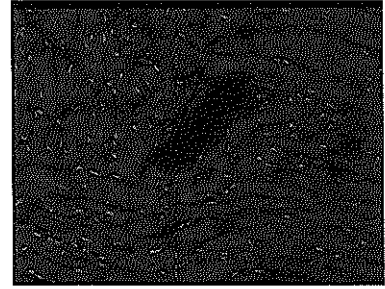
A



B



C



D

Discussion

The aim of our study was to compare the survival of single and double transgenic mouse hearts in the Langendorff circuit by means of ex vivo heart perfusion with human serum. Although it is important to find out which protein, or combination of proteins can be used to prevent HAR, there are remarkably few functional comparisons of complement regulatory proteins in the literature (12). Our transgenic mouse lines were analyzed by Southern blot analysis of genomic tail DNA (10;11), but also by immunohistochemistry and FACS analysis of splenocytes. We found that the entire genomic DNA encoding for human complement regulatory protein was successfully integrated into the mouse genome and that the proteins were expressed on the vascular endothelium. Mouse hearts transgenic for hCD55 or hCD46 perfused with human serum survived significantly longer than their non-transgenic controls, which confirms earlier results found by us and others (5;7;9). However, hCD59 hearts

survived as long as their controls. Others have found (13-18) that hCD55, hCD46 and hCD59 all give protection against HAR. The controversy about CD59 could firstly be explained by the fact that our hCD59 mice had 3 integrated copies of the entire DNA encoding for hCD59 and may therefore have a lower expression of the protein, resulting in a worse protection against HAR. Additionally, hCD55 and hCD46 had 10 and 10-12 integrated copies, respectively, and their expression may be higher, leading to a better protection against HAR. In addition, it is proven that CRPs are not that species-specific, but the amount of expression of the CRPs is important (19). Secondly, because of the wide range in beating times, our hCD59 mouse hearts just did not survive significantly longer than their controls, but a trend is present.

About the double transgenes we have found that hCD59xhCD55 and hCD59xhCD46 hearts beat longer as compared to non-transgenic controls, but not compared to single transgenes. Studies with hCD59xhCD55 transgenic mice (20) and pigs (21) support our results. To our knowledge, the hCD59xhCD46 combination has not been tested before.

hCD55xhCD46 hearts survived as long as their controls, and significantly shorter as compared to hCD55 or hCD46 single transgenic mice. In the literature one in vitro study could be found which reports that CD55 and CD46 synergize in preventing C3b deposition on the cell surface, and that the activities of CD55 and CD46, when present together, are greater than the sum of the two proteins individually (22). This finding is in contrast to our findings. Our result suggests that CD55 and CD46 have a negative effect on each other. We may hypothesize that, since there is no difference in expression level between the CD55 and CD46 proteins, the discrepancy between the single (hCD55, hCD46) and double (hCD55xhCD46) transgenic mice can be due to the fact that there is a functional hindrance between hCD55 and hCD46.

The immunohistochemical staining with antihuman C3, C9, IgA, IgG and IgM antibodies of all hearts did not give extra information. No difference in C3 and C9 was found probably due to the fact that staining was performed after cessation of heartbeats, i.e. rejection of the hearts, at the end of the perfusion period. This also holds for IgA, IgG and IgM depositions. IgA deposition can be found when the alternative pathway of the complement cascade is activated. IgM and IgG are present if the classical pathway is activated. In our model IgA, IgG and IgM antibodies reached similar levels. We might conclude that the classical and the alternative pathways both contribute to the activation of the complement system, leading to endothelial cell damage, represented by cessation of heartbeats.

We conclude that mouse hearts transgenic for hCD46 or hCD55 are protected against HAR, whereas hCD59 hearts are not. Furthermore, that hCD59xhCD55 and hCD59xhCD46 double

transgenic mouse hearts are better protected against HAR than their controls, but they are not better protected as compared to single transgenic mouse hearts (hCD55 or hCD46). However, the combination hCD55xhCD46 is even worse than hCD55 or hCD46 alone. In both single and double transgenic, and non-transgenic mouse hearts equal levels of antihuman C3c, C9, IgA, IgG, and IgM depositions have been found. The most important conclusion of our study is that it is enough to be transgenic for one complement regulatory protein, which inhibits at the level of C3, to prevent HAR.

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CHAPTER 5

CONTRAST IN THE EFFICACY OF hDAF MOUSE HEARTS BETWEEN EX VIVO PERFUSION AND TRANSPLANTATION INTO PRIMATES

C.A.E. VERBAKEL¹, R.W.F. DE BRUIN¹, F. BONTHUIS¹, M. JONKER²,
S. DEKKER³, R.L. MARQUET¹, J.N.M. IJZERMANS¹

¹LABORATORY FOR EXPERIMENTAL SURGERY, ERASMUS UNIVERSITY ROTTERDAM

²BIOMEDICAL PRIMATE RESEARCH CENTRE, RIJSWIJK

³LABORATORY FOR CELL BIOLOGY & GENETICS, ERASMUS UNIVERSITY ROTTERDAM

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Abstract

In recent experiments, in which we compared hDAF transgenic rat hearts perfused with 15% human serum in the Langendorff device and hDAF rat hearts transplanted into cynomolgus monkeys, we demonstrated that in the ex vivo heart perfusion model both homozygous and heterozygous hDAF hearts survived longer as non-transgenic controls. Surprisingly, we found that only homozygous hDAF hearts were protected against hyperacute rejection in vivo. The first aim of this study was to determine whether perfusion of mouse hearts with higher human serum concentrations or human blood might explain some of the differences found in survival time of the recently performed experiments with rat heart xenografts. Secondly, we investigated whether the observed differences in survival times of rat xenografts between in vivo and ex vivo transplantation would also hold for mouse hearts transgenic for hDAF. An ex vivo model was used to perfuse hDAF mouse hearts and controls with human serum or blood, and hDAF transgenic hearts and controls were transplanted into cynomolgus monkeys. hDAF transgenic mouse hearts survived significantly longer than their controls when perfused with 15% human serum, but no difference was found when 30% human serum was used, or when these hearts were transplanted into cynomolgus monkeys. However, in both in vivo and ex vivo model the amount of PMNs adhering to the vascular endothelium was significantly lower in hDAF transgenes as compared to their controls. In conclusion, in the ex vivo situation, the efficacy of hDAF transgenesis in preventing HAR is limited by serum complement concentration.

Introduction

As complement plays a dominant role in the process of hyperacute rejection (HAR) of xenografts, regulation of complement activation is required to overcome HAR. This can be accomplished by over-expression in the donor of, preferably, recipient-type complement regulatory proteins (CRPs), such as Decay Accelerating Factor (DAF, CD55), Membrane Cofactor Protein (MCP, CD46) or Protectin (CD59). Inhibition of HAR has been achieved using this transgenic approach with varying success, depending on the experimental model (1-9). The Langendorff perfusion system has been employed by us to evaluate the relative importance of human CRPs on the performance of mouse hearts perfused with human serum. In this *ex vivo* model of xenotransplantation, we showed that over-expression of human MCP (hCD46) or human DAF (hCD55), but not hCD59, protected hearts against hyperacute rejection (10-13).

Recently, we perfused hDAF transgenic rat hearts with human serum in the Langendorff device and the results were compared with the survival data of hDAF rat hearts transplanted into cynomolgus monkeys. Surprisingly, we found that both homozygous and heterozygous hDAF-hearts, when perfused with human serum in the *ex vivo* perfusion system, showed prolonged survival in contrast to control hearts, whereas *in vivo* only homozygous hearts were protected against HAR (14). This finding might be explained by the assumption that HAR of a xenograft is determined by the balance between protection at the site of the graft (i.e. the level of expression of hDAF) and the magnitude of the immunological complement-mediated assault provided by the recipient (i.e. complement levels). It may be speculated that a combination of various transgenic CRPs is more effective in protecting the xenogeneic graft. With the availability of multiple transgenic mice strains, we wanted to test and examine the predictive value of the *ex vivo* heart perfusion system for *in vivo* xenotransplantation first, before we studied the relevance of multiple transgenics in modification of xenogeneic rejection. Our main goal was to investigate whether serum concentration in relation to a certain level of hDAF expression in mouse hearts influences HAR and activation of endothelial cells leading to adherence of PMNs. And furthermore, to determine whether perfusion of mouse hearts with higher human serum dosages (tipping of the balance) or human blood (presence of cells) might explain some of the differences found in survival times of the recently performed experiments with rat heart xenografts.

Materials and Methods

Animals

Mice

Heterozygous and homozygous B₁₀CBA-mice transgenic for hDAF and their non-transgenic counterparts were used. The animals weighed approximately 20 grams and were twelve-to-sixteen weeks old. Production of these transgenic mice was accomplished by injecting mouse ova with the entire genomic DNA encoding for hDAF using yeast artificial chromosomes (YAC), which has been described by Yannoutsos et al. (13). The hDAF-mouse strain used, contained 10 integrated copies in the genome and the expression of the relevant protein on spleen, kidney, heart, liver and lung was verified by anti-hDAF (4). Splenocytes of mice transgenic for hDAF showed a strong expression of the transgene by FACS analysis. The animals were housed in a certified animal breeding facility under standard conditions and had free access to standard mouse chow (AM II, Hope Farms, Woerden, The Netherlands) and water acidified to pH 2.2 ad libitum. The experimental protocols adhered to the rules laid down in the Dutch Animal Experimentation Act (1977) and the published Guidelines on the Protection of Experimental Animals by the Council of the EC (1986). The Committee on Animal Research of Erasmus university Medical Centre, Rotterdam, The Netherlands, and that of the Biomedical Primate Research Centre (monkey experiments), Rijswijk, The Netherlands had given permission to perform the experimental protocols.

Cynomolgus monkeys

Two cynomolgus monkeys (*macaca fascicularis*) of the Biomedical Primate Research Centre (BPRC), Rijswijk, The Netherlands, were used as recipients. The first monkey was a five year old male with blood type AB, weighing 8100 grams and the second a six year old female with blood type A, weighing 4900 grams. The animals had been used for allogeneic skin transplantation experiments more than six months before the present study. Their hematological and immunological status was normal. The animals were housed in a certified animal facility at the BPRC (Rijswijk, The Netherlands) under standard conditions.

The Langendorff circuit

All mice were sedated with Diazepam (5 mg / kg i.m.) to reduce the escape risk. Prior to the surgical procedure, they were anaesthetised with iso-flurane inhalation (Pharmachemie B.V. Haarlem, Holland). The abdominal wall was opened and 0.6 mL Heparin (50 IU/mL) was injected into the abdominal inferior vena cava. Via two lateral incisions the thorax was

opened and the heart was removed quickly. A 22-gauge indwelling cannula was inserted into the aorta and secured with a 4.0 ligature. The heart was contiguously perfused with oxygenated Krebs-Henseleit solution (NaCl: 118.0mM, KCl: 4.70mM, CaCl₂: 2.52mM, MgSO₄: 1.66mM, NaHCO₃: 24.88mM, KH₂PO₄: 1.18mM, glucose: 5.55mM, Natriumpyruvate: 2.0mM (Merck, Darmstadt, Germany)) using a syringe and directly linked to the Langendorff device. The Langendorff circuit consisted of a roller pump, two double-layered glass reservoirs of 500 mL and 150 mL connected with the heart via a three-way cock, and a water column providing a perfusion pressure of 70 cmH₂O. Oxygenation was obtained by continuous gassing with a mixture of 95% O₂ and 5% CO₂ (carbogen) through 10 meters of silastic tubing, which was inserted into the tubing of the perfusion circuit, providing a pO₂ of 300 to 600 mmHg. A temperature of 37 °C of the perfusate solution was maintained by heated water running between the double-layered glass of the reservoirs and along the final part of the perfusion system. The largest reservoir was filled with a solution containing 30% Haemaccel and 70% Krebs-Henseleit buffer. Haemaccel was added to optimize the performance of the mouse hearts during perfusion. The small reservoir was filled with human serum or human blood diluted with the solution from the large reservoir. Before switching to human serum or blood, acclimatisation of the hearts was accomplished by perfusion with Krebs-Henseleit-Haemaccel solution for 10 minutes. Hearts were monitored constantly for heart rate and flow using multichannel registration. Survival time was defined as the time between the start of serum/blood perfusion and complete cessation of heartbeats.

Heart transplantation

The surgical procedure of removal of the heart was the same as described above, except that the mice were anaesthetised with ether.

The monkeys were pre-medicated with Ketamin (100 mg) and Atropin (0.4 mg), and intubated after administration of Xylocain spray. Iso-flurane inhalation was used as anaesthetic. During the operation infusion with NaCl + glucose 2.5% was given. The inguineal area of the monkey was opened and the left femoral artery was dissected. An arterial line (polyethylene tubing, diameter 1.4 mm) was inserted and fixed with a 4.0 ligature, at the end of this arterial line a three-way cock was attached. After flushing the line, heterotopic heart xenotransplantation was performed by connecting the 22-gauge cannula attached to the murine aorta (as described above) with the arterial line from the monkey. Only survival time was measured, which was defined as the time between the start of blood perfusion and complete cessation of heartbeats. Venous blood pumped from the mouse heart (about 3 mL per minute) was not recirculated into the monkey, but was collected in a jar. In

each monkey six transplantations were performed: three transgenic hearts and three controls, which sequentially were connected to the same cannula. After the procedure, the monkeys were euthanised.

Human serum and blood

Frozen, pooled, human O-type was obtained from the blood bank of the University Hospital Dijkzigt (Rotterdam, The Netherlands). Human serum was made out of human plasma by adding 0.5 mL Thrombin to 200 mL human plasma, inducing clotting. After removal of the clot, the serum was diluted with Krebs-Henseleit-Haemaccel solution to obtain perfusion concentrations of 15% and 30%. For each experiment fresh human serum was prepared.

O-type EDTA blood was collected in 9 mL test tubes containing EDTA at the blood bank and immediately processed as follows. After centrifugation (10 minutes, 2000 rpm), the plasma was removed and replaced by a similar volume of serum, which was prepared as described above. To obtain the 15% solution for perfusion, the blood was diluted with Krebs-Henseleit-Haemaccel stock solution. Finally, 0.1 mL heparin (50 IU/mL) was added to this diluted solution to achieve anticoagulation.

Experimental groups

The experimental groups consisted of hDAF transgenic hearts and their controls perfused with either 15% and 30% human serum, or 15% human blood. In addition, in two groups hDAF transgenic and control hearts were transplanted into cynomolgus monkeys.

Immunohistochemistry

After cessation of heartbeats each heart was cut in two. One sample was fixed in 3.6% formaldehyde solution. Following dehydration and paraffin embedding, 4 µm-thick sections were cut and stained with Hematoxylin & Eosin (H&E). The other part of the sample was snap frozen and stored at -80 °C until sectioned for immunohistochemistry (C3c and C9). Frozen tissue sections (7 µm-thick) were prepared in a Microm HM 560 cryostat and stained with rabbit anti-human C3c complement Fluorescein-Iso-Thio-Cyanate (FITC) and rabbit anti-human C9.

C3c staining: the frozen sections were thawed at room temperature for 1 hour, fixed with acetone for 10 minutes and air dried for 15 minutes. With a Peroxidase-Anti-Peroxidase(PAP)-pen circles were drawn around the tissue sections and washed with PBS 0.1% BSA three times. Diluted (1:160) anti-human C3c FITC-antibody was added to the

sections and incubated for 60 minutes in wet surroundings. The abundant antibody was washed away with water and the sections were put in Mayer's hematoxylin for 40 seconds and secondly in an ammonia-solution. After dehydration, the sections were put in Xylene. The slides were covered with Entalan.

C9 staining: the frozen sections were thawed at room temperature for 1 hour, fixed with acetone for 10 minutes and air dried for 15 minutes. With a PAP-pen circles were drawn around the tissue sections and washed with PBS 0.1% BSA three times. Sections were incubated for 5 minutes with peroxidase block (Envision kit) in wet surroundings and cleanse three times with PBS. Diluted (1:400) anti-human C9-antibody was added and the sections were incubated for 30-60 minutes in wet surroundings. Again sections were washed three times with PBS. Sections were incubated with 'labelled polymer-HRP' (Envision kit) for 30 minutes and washed three times with PBS. Diaminobenzidine (DAB) (Envision) was added and sections were incubated for 5-15 minutes. The DAB-reaction was stopped by flushing with water for 5 minutes. Sections were put in Mayer's haematoxylin for 40 seconds, washed, and were then put in an ammonia-solution. Dehydration was followed by a Xylene step. The slides were covered with Entalan.

Anti-human C3c-stained sections and anti-human C9-stained sections were examined under a fluorescence microscope and a light microscope, respectively. The intensity of C3c- and C9-expression on the heart tissues was scored blindly via semi-quantitative analysis: 0 = no staining, 0.5 = mild staining, 1 = dense staining.

All transgenic and non-transgenic mouse hearts of both the monkey and Langendorff model were stained for H&E, C3c and C9. Using H&E stained sections, the transplanted transgenic and non-transgenic hearts were examined for edema, hemorrhage, microthrombosis and polymorphonuclear (PMN) cell adhesion. In twenty vessels the amount of PMN-cells adhering to the vessel wall and the amount of cells lying intraluminally was counted. The percentage of cells adhering to the vessel wall was calculated. This was also done for the transgenic and non-transgenic hearts perfused with human blood in the Langendorff device.

Statistical analysis

The non-parametric Mann-Whitney test was used to analyse the differences in survival times between the experimental groups. The difference between the experimental groups in percentage PMN-adhesion and C3c-deposition was done by using the Student's t-test. The level of significance being $P < .05$.

Results

Langendorff perfusions

Survival times

When heat-inactivated human serum is used for perfusion, the hearts kept on beating until 45-60 minutes (data not shown). The results obtained in the experimental groups are summarised in table 1. Control hearts perfused with 15% human serum had a mean survival time (MST) of 4.4 minutes, whereas transgenes showed a MST of 24 minutes ($P = .006$). The survival times following perfusion with 30% human serum were shorter than after perfusion with 15% serum for both the controls and the transgenic hearts (MSTs of 1.0 and 1.5 minutes with P -values of .003 and .005, respectively). Perfusion of control hearts with 15% human blood led to a better performance than perfusion of control hearts with serum, leading to a MST of 17 minutes (group 1 versus group 5, $P = .065$). Transgenes perfused with blood performed similarly as blood-perfused controls: MST of 12 minutes ($P = .223$).

The mean heart rate for both hDAF transgenes and controls measured by multichannel registration was 100 beats / min. After switching to human serum or blood, it remained the same for hDAF transgenes and decreased slightly in the control group to 90 beats / min. During the 10 minutes perfusion with Krebs-Henseleit the flow through transgenic and non-transgenic hearts remained constant at a mean level of 7 mL / min. After switching to human serum the flow lingered. However, when switched to human blood, the flow decreased to a mean level of 3 mL / min. The flow became 0 mL / min when the hearts stopped beating.

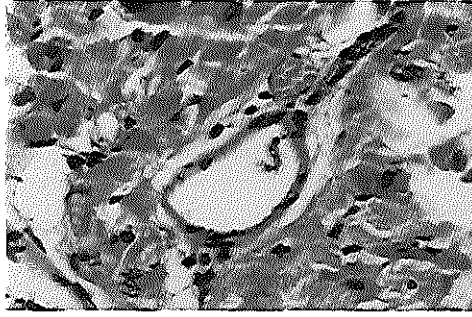


Figure 1. A medium sized vessel of a non-transgenic heart with “hairy” endothelium (H&E, 400x). HAR is caused by xenogeneic natural antibody and complement depositions, resulting in activation of endothelial cells. The major features of HAR in vivo are platelet aggregation and adhesion, fibrin deposition, interstitial edema, hemorrhage, PMN adhesion, vasoconstriction and ‘hairy’ endothelium. In the Langendorff ex vivo perfusion system these features are not prominent.

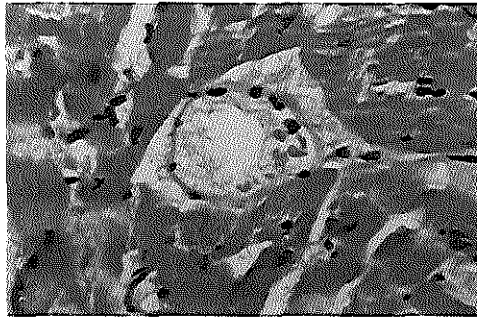


Figure 3. A medium sized vessel of a non-transgenic heart which was transplanted into a primate (H&E, 400x). Myocardial edema, hemorrhage and micro-thrombosis, characterised by fibrin deposition and deposition of erythrocytes and platelets can be seen. Furthermore, PMN infiltration and adhesion was observed.

Table 1. Survival times and mean survival times (MST) of hDAF transgenic mouse hearts and their controls perfused with 15% and 30% human serum (HS), and 15% human blood (HB) in the Langendorff system. Three hDAF transgenic mouse hearts and three controls were transplanted into each monkey.

Mouse hearts + 15% HS	Survival time (minutes)	MST (minutes)
Group 1: B ₁₀ CBA (n=5)	3-4-4-5-6	4.4
Group 2: hDAF (n=8)	5-15-22-29-30-30-30-30	24
Mouse hearts + 30% HS		
Group 3: B ₁₀ CBA (n=6)	1-1-1-1-1-1	1.0
Group 4: hDAF (n=4)	1-1-2-2	1.5
Mouse hearts + 15% HB		
Group 5: B ₁₀ CBA (n=13)	1-3-4-5-7-10-17-28-30-30-30-30-30	17
Group 6: hDAF (n=8)	1-2-2-3-5-24-30-30	12
Mouse hearts to monkeys	Survival times (minutes)	MST (minutes)
Group 7: B ₁₀ CBA (n=6)	4-6-8-9-9-13	8.2
Group 8: hDAF (n=6)	5-6-9-11-12-13	9.3

P-values of table 1:

group 1 versus group 2 (P=.006), group 1 versus group 3 (P=.003), group 3 versus group 5 (P=.001), group 2 versus group 4 (P=.005), group 1 versus group 7 (P=.033), group 2 versus group 8 (P=.016), group 3 versus group 7 (P=.002), group 4 versus group 8 (P=.01).

Histology

Microscopic examination of H&E stained sections of the transgenic and non-transgenic hearts perfused with human serum showed normal cell architecture of the cardiomyocytes; “hairy” endothelium was only seen in rejected non-transgenic hearts (figure 1). The major features of HAR in vivo are platelet aggregation and adhesion, vasoconstriction, fibrin deposition, interstitial edema, hemorrhage, PMN adhesion and ‘hairy’ endothelium. In the Langendorff ex vivo perfusion system these features are not prominent. H&E sections of both non-transgenic and transgenic hearts perfused with human blood showed edema, hemorrhage and presence of PMNs. A difference in percentage PMN-adhesion to the vascular endothelium was found: transgenic hearts (46 ± 12%) showed a significantly lower amount of PMN-adherence than non-transgenic hearts (73 ± 9%) (P = .0001).

Complement deposition

C3c-levels of groups 1 and 2 in relation to survival time showed that in both groups rejection is not necessarily related to C3c deposition. In the controls 2 out of 4 rejected hearts showed no C3c deposition, whereas 2 showed a marked deposition. In the transgenic hearts 2 out of 6 were negative and 4 showed moderate to strong C3c deposition. In figure 2 C3c-levels are given as observed in hearts perfused with 15% human blood. Here, controls showed significantly more deposition of C3c than transgenic hearts (mean scores of 0.65 and 0.25, respectively; $P < .05$). Light microscopical examination of tissues stained for anti-human C9 antibodies, which were perfused with human serum and blood, exhibited no difference in amount of deposition of C9. In both non-transgenic and transgenic hearts C3c and C9 depositions were mainly localised at the vascular endothelium and diffuse along the cardiomyocytes.

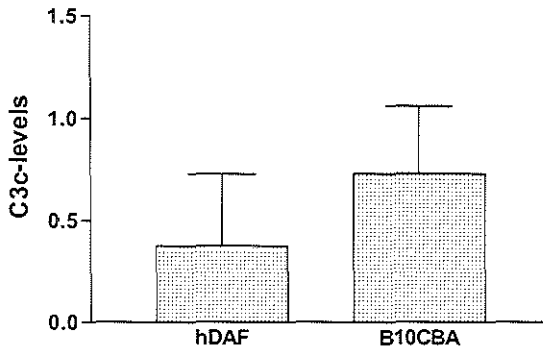


Figure 2. C3c-levels in hDAF transgenic mouse hearts and controls perfused with 15% human blood. C3c was mainly localised at the vascular endothelium and diffuse along the cardiomyocytes. On the x-axis the experimental groups are given. The y-axis depicts the semi-quantitative C3c-levels with 0 = no, 0.5 = mild and 1 = dense staining.

Mouse-to-monkey heart transplantation

Survival times

The data from the two monkeys were pooled. Each monkey received control and hDAF hearts. The survival times obtained in groups 7 and 8 are given in table 1. There was no significant difference in survival times ($P = .517$); controls had a MST of 8.2 minutes; transgenic hearts had a MST of 9.3 minutes.

Histology

Both transgenic and non-transgenic hearts exhibited myocardial edema. In controls hemorrhage and micro-thrombosis, characterised by fibrin deposition and deposition of erythrocytes and platelets, was seen (figure 3). Furthermore, PMN infiltration and adhesion was observed. Rejected transgenic hearts showed less hemorrhage, less micro-thrombosis, and the amount of PMNs adhering to the vessel wall was significantly lower than in controls: $59 \pm 11\%$ in controls versus $32 \pm 6\%$ in hDAF transgenics ($P = .000$). Both transgenic and non-transgenic mouse hearts showed a similar diffuse deposition of C3c and C9 on the vascular endothelium and cardiomyocytes.

Discussion

HAR is the major cause of organ failure in discordant xenogeneic transplantation models, and is caused by activation of the complement, binding of xenoreactive natural antibodies (XNA) to the donor endothelium and by blood cell-endothelial cell interactions. Complement is normally controlled by regulatory factors. Manipulation at the level of complement is a manner to prevent HAR. To this end, DAF-, MCP- and CD59-transgenic donor animals (mouse/rat/pig) have been generated and tested in *ex vivo* and *in vivo* models. In our study, one of our aims was to determine whether the intensity of endothelial cell activation leads to activation of PMNs and clotting, and if, at a certain hDAF expression, the process was serum dosage dependent. We found that hDAF mouse hearts were markedly protected from HAR when perfused with 15% human serum, whereas such protection was not observed when these hearts were transplanted in cynomolgus monkeys. However, in the latter model we did observe other relevant differences between transgenic and non-transgenic hearts. Transgenic hearts showed less hemorrhage and thrombosis and fewer PMNs adhering to the vascular endothelium as compared to non-transgenic controls. Apparently, this protection was not sufficient to produce prolonged survival in a non-immunosuppressed monkey. In contrast, we have earlier found differences in survival times between hDAF transgenic rat hearts and their controls transplanted into non-immunosuppressed monkeys (14), and in only one study, in which two hDAF/hCD59 transgenic porcine cardiac xenografts and their controls were transplanted into non-immunosuppressed baboons, controls rejected in 60-90 minutes and transgenic xenografts survived for 6 and 69 hours (15). In our primate model, we did not observe a difference in xenograft survival. This might be due to the amount of complement present in the blood and the number of CRPs present on transgenic vascular endothelium.

The next objective of our study was to see whether the differences between ex-vivo and in vivo might disappear when the in-vivo situation was mimicked. This was done 1) by increasing the human serum concentration from 15% to 30% and 2) by perfusion with human blood. And indeed, the protective effect of hDAF seemed to vanish. The mean survival times of both transgenic and non-transgenic mouse hearts perfused with 30% human serum were not significantly different. These results indicate that the efficacy of transgenesis is related in a dose-dependent manner with the human serum concentration. Consequently, a relative excess of complement abolishes the protective effect of CRPs. This dysbalance between complement effectors and inhibitors leads to an overwhelming complement-mediated attack with rapid endothelium destruction, resulting in a short graft survival time. hDAF did not protect against HAR when hearts were perfused with 15% human blood, despite of a low serum concentration. In our model, it was observed that hDAF had a significant inhibitory effect on the adhesion of leukocytes to the vascular endothelium and deposition of C3c. This may be explained by the following theory: once the complement cascade is activated, the anaphylatoxins C3a and C5a, are released, resulting in chemotaxis of leukocytes to inflammatory sites, where they produce cytokines and mediate the inflammatory response. Of the various membrane-associated complement inhibitors, DAF, MCP and CR1 may be the most useful because they prevent generation of the anaphylatoxins C3a and C5a, as well as the membrane attack complex (16;17). Furthermore, a lower amount of adhering leukocytes in transgenic mouse hearts was found, because the C3-pathway of the complement cascade is blocked by hDAF, which leads to lower levels of C3a and C5a, and consequently to less chemotaxis and leukocyte adherence. In the literature this theory is supported by other research groups: expression of DAF should decrease the production of C5a and may reduce the degree of neutrophil infiltration into the organs (15); CD55 (DAF) reduces the generation of C3a, C5a, iC3b, and C5b-7, all of which have deleterious effects on graft survival (17), and activation of complement on endothelial surfaces promotes neutrophil adhesion (18;19).

In summary, in our model, ex-vivo human serum perfused mouse hearts expressing hDAF were protected against HAR, whereas in vivo hDAF mouse hearts were not. Differences in graft survival were only found, when perfusion with a low serum concentration was employed. Using a higher serum concentration or blood, no differences in survival times were found, but differences in edema, hemorrhage, complement depositions and PMN adherence did occur. The Langendorff perfusion model allows evaluation of CRPs, but only in the early stages of complement-mediated injury, including the adherence of PMNs.

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CHAPTER 6

PROTECTION AGAINST HYPERACUTE XENOGRFT REJECTION OF TRANSGENIC RAT HEARTS EXPRESSING HUMAN DECAY ACCELERATING FACTOR (DAF) TRANSPLANTED INTO PRIMATES

B. CHARREAU¹, S. MÉNORET¹, L. TESSON¹, A. AZIMZADEH², M. AUDET²,
PH. WOLF², R.L. MARQUET³, C.A.E. VERBAKEL³, J.N.M. IJZERMANS³,
P. COWAN⁴, M. PEARSE⁴, A. D'APICE⁴, JP. SOULLILOU¹, I. ANEGON¹

¹INSERM U437 IMMUNOINTERVENTION EN ALLO ET XÉNOTRANSPLANTATION AND INSTITUT
DE TRANSPLANTATION ET DE RECHERCHE EN TRANSPLANTATION, NANTES, FRANCE

²FONDATION DE TRANSPLANTATION, STRASBOURG, FRANCE

³LABORATORY FOR EXPERIMENTAL SURGERY, ERASMUS UNIVERSITY ROTTERDAM

⁴IMMUNOLOGY RESEARCH CENTER, ST. VINCENT'S HOSPITAL, FITZROY, VICTORIA, AUSTRALIA

Abstract

Background. Production of transgenic pigs for multiple transgenes is part of a potential strategy to prevent immunological events involved in xenograft rejection. Use of a genetically engineerable rodent as a donor in primates could allow testing in vivo of the effects of different transgenes on controlling xenograft rejection. As a first step in the development of a donor containing multiple transgenes, transgenic rats for human decay accelerating factor (DAF) were used as heart donors to test their resistance against complement (C)-mediated rejection by non-human primates.

Materials and Methods. Transgenic rats were generated by using a construct containing the human DAF cDNA under the transcriptional control of the endothelial cell (EC)-specific human ICAM-2 promoter. DAF expression was evaluated by immunohistology and by FACS analysis of purified ECs. Resistance of transgenic hearts against C-mediated damage was evaluated by ex vivo perfusion with human serum and by transplantation into cynomolgus monkeys.

Results. Immunohistological analysis of DAF expression in several organs from two transgenic lines showed uniform expression on the endothelium of all blood vessels. ECs purified from transgenic hearts showed 50% DAF expression compared to human ECs, and >70% reduction of C-dependent cell lysis compared to control rat ECs. Hemizygous transgenic hearts perfused with human serum showed normal function for >60 min vs. 11.2 ± 1.7 min in controls. Hemi- or homozygous transgenic hearts transplanted into cynomolgus monkeys showed longer survival (15.2 ± 7 min and >4.5 hr, respectively) than controls (5.5 ± 1.4 min). In contrast to hyperacutely rejected control hearts, rejected homozygous DAF hearts showed signs of acute vascular rejection (AVR) characterized by edema, hemorrhage, and an intense PMN infiltration.

Conclusions. We demonstrate that endothelial-specific DAF expression increased heart transplant survival in a rat-to-primate model of xenotransplantation. This will aid in the analysis of AVR and of new genes that may inhibit this form of rejection, thus helping to define strategies for the production of transgenic pigs.

Introduction

The use of porcine organs for clinical transplantation is an approach in overcoming the shortage of human organs. The first obstacle to xenotransplantation is the recipient's innate immune response which results in xenograft hyperacute rejection (HAR) (1). Rejection of transplants performed between discordant species, such as pig-to-primates, involves the binding of preformed xenogeneic natural antibodies (XNA) directed against Gal α 1-3Gal epitopes on the endothelial cell (EC) surface (2;3). Interaction between XNA and their targets promotes complement (C) activation, leading to graft endothelium injury and activation (4-7). Prevention of xenograft HAR has been successfully achieved by either inhibition of XNA binding through depletion of XNA or reduction of Gal α 1-3Gal expression on ECs (8;9), or blockade of complement activation (10). Transgenic pigs expressing human complement regulatory proteins (CRPs), including decay accelerating factor (DAF) and/or CD59, have been produced to avoid complement-mediated graft damage (11-13). Dramatic improvement of xenograft survival has been obtained using these transgenic transplants in the pig-to-primate model of xenotransplantation (14-16). These studies demonstrated that human DAF and/or CD59 expression on graft ECs, in association with antibody depletion and/or immunosuppressive regimen, efficiently overcome HAR. However, optimal transgene expression levels as well as its optimal tissue distribution remained to be determined.

Since xenograft HAR can be circumvented by the use of transgenic animals for CRPs, humoral and cell-mediated processes involved in delayed xenograft rejection or AVR may be further controlled by the production of transgenic animals expressing other transgenes acting on these processes in addition to CRPs. To this end, prior to the generation of transgenic pigs, production of transgenic small laboratory animals is needed. These animals would not only serve as tools for analysis of DNA construction for transgenesis but more importantly would help us investigate *in vivo* the biological relevance of transgene expression on xenograft rejection. In addition, models bearing close resemblance to the pig-to-primate one may be particularly useful. In this regard, although transgenic mice have been used to define the expression of DNA construction (17;18) and their organs perfused *ex vivo*, their reduced size make it nearly impossible to graft their organs in primates.

We previously established an *in vivo* experimental model for xenotransplantation using the rat as organ donor and an Old World primate (cynomolgus) as recipient. In this rat-to-primate combination, HAR occurred 5 min after vascular anastomosis, with features mimicking HAR (19) and EC injury (7) observed in the pig-to-primate model. In addition, we demonstrated that, *in vitro*, human DAF and CD59 efficiently protect transfected xenogeneic rat ECs against cell lysis mediated by non-human primate sera (20). In the present study, we

investigated the ability of human DAF expressed by the endothelium to delay HAR by producing transgenic rats for the human DAF. Endothelium-specific transgene expression was analyzed by immunohistochemistry in tissue sections and quantitated in vitro by isolation of cardiac ECs followed by flow cytometry. Ex vivo perfusion of transgenic hearts with human serum demonstrated the ability of human DAF expression to prevent XNA and C-mediated organ damage. Correlation between the DAF expression level on graft ECs and graft survival time was documented following cardiac heterotopic transplantation using hemizygous and homozygous transgenic rats as donors and unmodified cynomolgus as recipients. Histopathological features of acute vascular rejection (AVR) of DAF-expressing cardiac xenograft were characterized by polymorphonuclear leukocytes (PMN) infiltration, interstitial edema, and focal myocardium necrosis.

Materials and Methods

Generation of transgenic rats

The plasmid expression vector pHICAM2DAF (figure 1A) contains a 1.9 kb *EcoRI* fragment of the human CD55 cDNA, 334 pb of the promoter region of the human intercellular adhesion molecule 2 (ICAM2) gene (nucleotides -292 to +44), a universal intron, and the SV40 polyA, as previously described (21). The 3.12 kb *KpnI-SacI* fragment was excised from pHICAM2DAF and isolated by electrophoresis through a 1% agarose gel, electroeluted, purified through an Elutip column (Schleicher & Schull, Keene, NH), and diluted to a concentration of 2 ng/ml in 5 mM Tris-HCl and 0.1 mM EDTA, Ph 7.4. Fertilized rat eggs were recovered from superovulated Sprague Dawley (SD) females mated with SD males (Charles River, Saint-Aubin-Les-Elbeuf, France). DNA solution (2 ng/ μ l) was microinjected into the male pronucleus and transferred into both oviducts of day 0 pseudopregnant SD females as previously described (22).

Identification of the transgenic founder animals and their progeny was determined by polymerase chain reaction (PCR) analysis of genomic DNA obtained from tail biopsies digested with proteinase K in total volume of 450 μ l for 16 hr at 56°C and then confirmed by Southern blots. PCR analysis was performed on 2 μ l of the DNA solution diluted to 1/20 with oligonucleotides specific for human DAF (sense: 5'GAC GCT AGT AAT CAT GGG CT3'; antisense: 5'TAG GAA AGG AAT CAC TCT CA3') for 30 cycles of amplification (94°C for 30 sec; 50°C for 30 sec; 72°C for 30 sec). For Southern blot analysis, 10 μ g of DNA was digested by *Bam*HI, fractionated on 1% agarose gel, and alkali blotted. Hybridization was carried out with a 32 P-dCTP-radiolabeled 3.12 kb *KpnI-SacI* fragment of the pHICAM2DAF

construct used for microinjection as a probe (probe showed in figure 1A). Determination of transgene copy number in genomic DNA from progeny and discrimination between hemizygous and homozygous rats was evaluated by probing a Southern blot of *Bam*HI-digested tail DNA and known quantities of the microinjected fragment diluted in genomic DNA, followed by PhosphorImager analysis (Molecular Dynamics, Sunnyvale, CA).

Endothelial cell (EC) isolation and culture

ECs were isolated from heart and lung. Briefly, tissues collected on transgenic or nontransgenic animals were cut in small pieces and then incubated with 1.8 U/ml Dispase II (Boehringer Mannheim, Mannheim, Germany), 0.5 mg/ml DNase I (Boehringer Mannheim) and 1% fetal calf serum (FCS) (Gibco BRL, Grand Island, NY) in Hank's buffered salt solution (HBSS) (Gibco BRL) for 4 hr at 37°C. Biopsies were then incubated overnight in 2 IU/ml collagenase B (Boehringer Mannheim) at 4°C. Cell suspension was washed three times in HBSS medium before being plated on gelatin-coated tissue culture dishes (Nunc, Naperville, IL). DAF-expressing porcine endothelial cells were isolated from transgenic pig aortas by collagenase digestion (1 U/ml; Boehringer Mannheim). Human umbilical vein endothelial cells (HUVEC) were prepared as previously described (23). Purity of EC preparations was checked by the uptake of Dil-Ac-LDL as previously described (20). Endothelial cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/liter glucose (Gibco BRL) and supplemented with 20% serum [fetal calf serum (FCS) (Gibco BRL) and human serum for rat ECs and HUVECs, respectively], 5 IU/ml heparin, 200 µg/ml Endothelial Cell Growth Supplement (Collaborative Biomedical Research, Becton Dickinson, San Jose, CA), 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco BRL). Cells were analyzed between passages 1 and 4.

FACS analysis

Endothelial cells ($1-2 \times 10^5$ cells/sample) were suspended with trypsin-EDTA (Gibco BRL), washed twice with phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and 0.01% NaN₃, and then incubated on ice for 30 min with a saturating concentration of the relevant monoclonal antibody (MAb). This step was followed by three washes in cold 1% BSA/0.1% NaN₃/PBS. Staining with a FITC-labeled-F(ab')₂ fragment of either goat anti-mouse, sheep anti-rabbit, or mouse anti-human immunoglobulin G (IgG) or IgM (Jackson Lab., West Grove, PA) was performed at 4°C for 30 min. Three washes in 1% BSA/0.1% NaN₃/PBS were performed before fixing the cells in 1% paraformaldehyde in PBS. The following antibodies were used in this study: rabbit polyclonal serum anti-human von

Willebrand factor (vWf) (Dako, Trappes, France), anti-human DAF (1H4) MAb (CRTS, Nantes, France), and anti-rat ICAM-1 MAb (Seikagaku America, Rockville, MD). For analysis of human Ig binding on rat ECs, cells were incubated with purified human anti-Gal α 1-3Gal antibodies (10 μ g/ml, 100 μ l/2 x 10⁵ cells) for 30 min at 4°C, washed, and then incubated with specific FITC-labeled goat F(ab')₂ anti-human IgM (Fc μ) or IgG (Fc γ) (Jackson Laboratories) antibodies.

Analysis of complement activation was investigated following incubation of rat ECs with human serum (dilution 1:10) in DMEM medium for 30 min at 37°C. Immunostaining was performed using FITC-labeled anti-human factor B, C3, C4 MAb (Atlantic Antibodies, Stillwater, MN) and an anti-human C5b-9 polyclonal serum as first antibodies revealed using FITC-labeled goat anti-rabbit IgG or IgM (Jackson Lab). Fluorescence was measured on 10,000 cells per sample using a FACScalibur (Becton Dickinson). Data are depicted in histograms plotting mean fluorescence intensity (MFI) on a log scale (x-axis) versus cell number (y-axis).

Complement-dependent cytotoxicity assay

Cells were labeled with ⁵¹Cr (50 μ Ci/1 x 10⁶ cells) for 1 hr at 37°C. Protection of DAF-expressing cells was assessed by incubating ⁵¹Cr-labeled rat ECs (2 x 10⁴ cells) with cynomolgus primate serum (as a source of xenogeneic antibodies and complement) (100 μ l, dilution 1:4 to 1:16) for 4 hr at 37°C. Chromium release was measured in supernatants, and the percentage of cell lysis was calculated by the following formula: (experimental cpm - cpm medium only)/(100% lysis cpm - cpm medium only). All experiments were done in triplicate, and the results are expressed as the mean of the percentage of specific lysis \pm SD values.

Immunohistochemistry

Tissues for immunohistology were included in OCT compound (Miles Laboratories, Elkhardt, IN) snap-frozen in precooled isopentane, and stored at -70°C until use. Human placenta and tissues from nontransgenic rats were included in every experiment as positive and negative controls, respectively. Frozen 5- μ m tissue sections were acetone fixed, hydrated with PBS, incubated with hydrogen peroxide at 0.15% in methanol for 20 min, blocked with rat serum at 10% in PBS/1% BSA, and incubated at room temperature for 60 min with MAb at 10 μ g/ml. Tissue sections were then incubated with biotin-conjugated horse anti-mouse IgG absorbed with rat serum proteins (Vector, Burlingame, CA) followed by horseradish peroxidase streptavidin (Vector) and developed with very intense purple (VIP kit, Vector). Slides were

then counter stained with hematoxilin and mounted with glycerol. The following antibodies were used in this study: anti-rabbit vWf, anti-human DAF (1H4), and purified human anti-Gal α 1-3Gal antibody. Negative controls were performed using an isotype-matched irrelevant MAb (3G8). Semi-quantitative analysis of DAF expression on endothelium was obtained by visual evaluation of the intensity of specific staining, which was ranked from 0 to 4 (0, no staining; 1, low intensity; 2, medium intensity; 3, high intensity; 4, very high intensity). Cardiac xenografts were analyzed for complement and xenogeneic antibody binding by immunofluorescence. Cynomolgus Ig binding was revealed through FITC-labeled goat F(ab')₂ anti-human IgM (Fc μ) or IgG (Fc γ) (Jackson Laboratories) antibodies.

Ex vivo perfusion of transgenic hearts with human serum

Rats were anesthetized with ether and the hearts were removed rapidly. A 22-gauge indwelling cannula was introduced into the aorta and secured with a ligature. Using a syringe, the hearts were perfused immediately with oxygenated perfusate and subsequently connected to Langendorff circuit. The circuit was oxygenated by continuous gassing with a mixture of 95% O₂ to 5% CO₂. The perfusate was kept at 37°C by continuous heated water. The largest reservoir was filled with a solution containing 30% Haemaccel (Behring, Marburg, Germany) in Krebs-Henseleit buffer (in mM: NaCl 118.0, KCl 4.7, CaCl₂ 2.52, MgSO₄ 1.66, NaHCO₃ 24.88, KH₂PO₄ 1.18, glucose 5.55, sodium pyruvate 2.0). The small reservoir was filled with 15% human serum (a pool of 20 human O-type plasma) diluted with the solution from the large reservoir. Before serum perfusion, hearts were allowed to stabilize by perfusing them with the Krebs-Henseleit-Haemaccel solution for 10 min. The hearts were monitored continuously for heart rate, apex amplitude, and flow by means of multichannel registration. Duration of heart function was defined as the time between the start of serum perfusion and complete cessation of heartbeats. Decomplementation was performed by heating of the serum for 1 hr at 56°C. Hemizygous rats (n = 5) from the transgenic line 52.2 were tested and compared to wild-type (WT) rats (n = 5).

Cardiac xenografts transplanted into primates

Transgenic and WT Sprague Dawley rats of 300-400 g body weight were used as donors and cynomolgus monkeys of 6-9 kg body weight were used as recipients. Heterotopic heart xenotransplantation was performed as previously described (19). To compare graft survival of hemizygous and homozygous transgenic hearts, one of each organ was concomitantly grafted onto the same recipient in each femoral vessel. Xenograft survival was determined by visual examination and palpation of the xenogeneic heart beating. Histological analysis of rejected

xenografts was performed on paraffin-embedded sections (5- μ m) stained with hematoxylin-eosin.

Statistical analysis

All data (mean \pm SD) were analyzed using the Student's *t*-test, with $P < .05$ being the level of significance.

Results

Production of human DAF transgenic rats and transgene integration analysis

A total of 754 eggs was microinjected and subsequently reimplanted into 37 pseudopregnant foster mothers, 27 of whom gave birth to 145 rats. Transgene integration was first detected by PCR in six founder animals and then confirmed by Southern blot performed on genomic DNA. Figure 1 shows the presence of human DAF sequence in a major restriction fragment of 3.1 kb in these transgenic lines as determined by hybridizing Southern blots, using the ICAM2DAF cDNA construct as a probe. The hybridization pattern revealed that multiple copy numbers of transgenes, in a head-to-tail orientation, had been integrated in all of these animals within one integration site, with the exception of line 70.8. Evaluation was done by comparing the size and intensity of hybridized fragment with known amounts of the microinjected fragment. High copy numbers (10 to 100) were observed in transgenic rats 58.3, 62.3 and 70.8. The transgene was transmitted to about 50% of the progeny for founders 52.2, 52.4, 62.3 and 70.8 (37%, 37%, 50%, and 50%, respectively). In contrast, a low transmission rate of the transgene to the offspring was obtained with founders 54.9 (8%) and 58.3 (<3%), which suggests that the germ-line in these founders was mosaic for DAF integration.

Tissue expression of human DAF in transgenic rats

Expression of human DAF was investigated in several organs from transgenic rats, including heart, kidney, liver, and lung, and on lymphoid organs (thymus, spleen). Cryosections of tissues were analyzed by immunohistology with an anti-human DAF MAb and compared to tissues from nontransgenic control rats. Consistent levels of DAF were detectable in tissues on progeny from lines 52.2 and 62.3. A low level of DAF was observed on line 52.4, while no expression was obtained for the lines 54.9, 58.3 and 70.8. For both transgenic lines (52.2 and 62.3), ECs expressing the transgene were detected in all analyzed tissues, including heart,

kidney, lung, and liver. In these organs, DAF expression was restricted to both vascular (large and medium size vessels) and capillar endothelium as determined by comparison with the pattern of staining obtained with an antibody directed to vWf (data not shown). All tissues analyzed from rat 52.2 showed higher expression of human DAF than those in corresponding organs from line 62.3. The nontransgenic (WT) control rats showed no staining of the tissues analyzed. Specific stainings for human DAF obtained in heart and kidney from an hemizygous transgenic rat (line 52.2) are presented in figure 2. Confirmation of endothelium-specific DAF expression in transgenic hearts was achieved by comparison with the pattern of staining obtained with purified human antiGal α 1-3Gal (7). Relative levels of DAF expression on cells from these tissues were compared and are summarized on figure 3. Expression was seen in a very small fraction of spleen cells in the white pulp. Similarly, a small fraction of medular thymic cells with dendritic prolongations showed expression of the transgene. High levels of expression were also detected in a fraction of granulocytes (7-10%) after indirect immunofluorescence labeling of leukocytes, red cells, and platelets followed by flow cytometry analysis (data not shown).

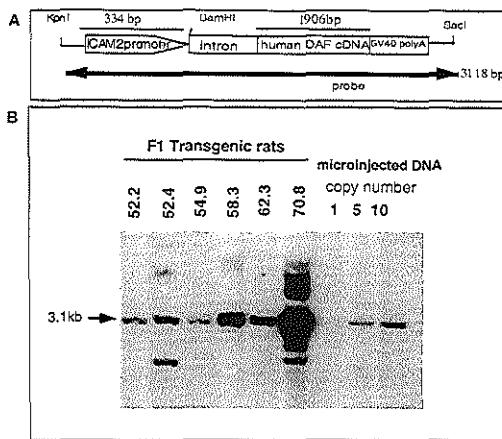


Figure 1. Analysis of transgene integration in transgenic rats. (A) Design of ICAM2DAF cDNA construct used for microinjection. (B) Southern blot from transgenic rats (DAF^{+/+}). Genomic DNA (10 μ g) was digested by *Bam*HI, separated through 1% agarose gel electrophoresis, and blotted. Known copy numbers of the microinjected fragment diluted into genomic DNA from a non-transgenic rat were used as controls. Blot was hybridized with a ³²P-dCTP labeled cDNA probe encoding the microinjected cDNA sequences (3.1 kb). *Bam*HI releases a 3.1 kb fragment (arrow).

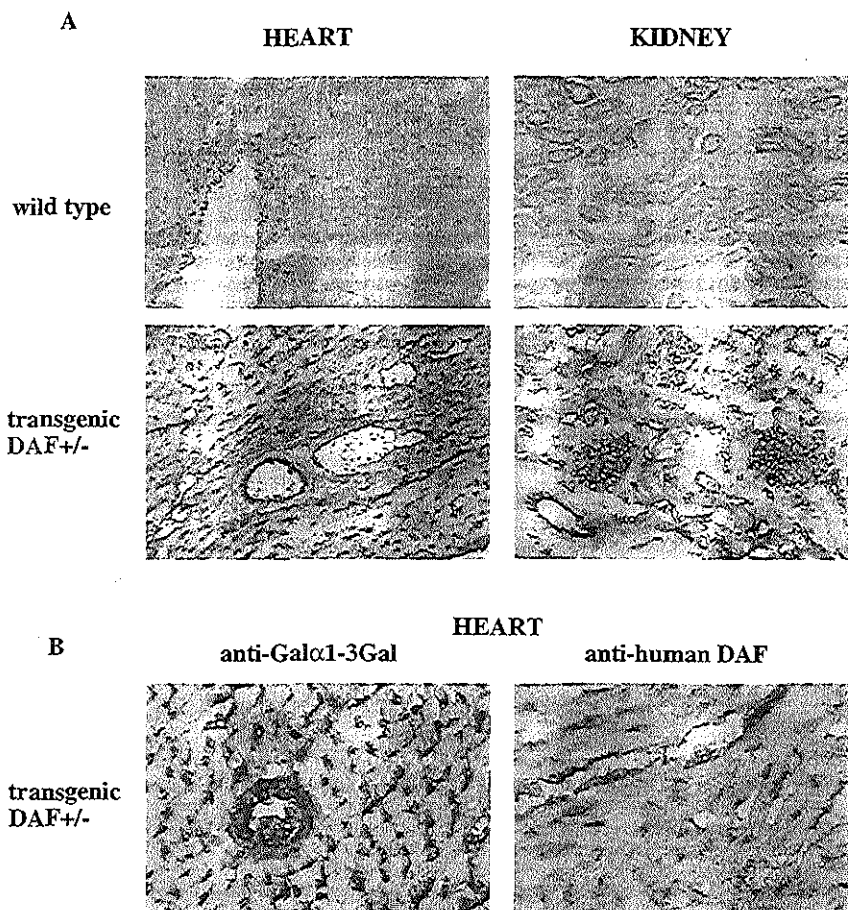


Figure 2. Expression of human DAF in transgenic rat tissues. Frozen sections (5 μ m) from transgenic (hemizygous DAF^{+/-} rat from line 52.2) or wild type (WT) rat tissues were fixed in acetone for 10 min at room temperature, air-dried, and stained using an indirect immunoperoxidase technique. (A) Immunostaining of serial tissue sections from kidney or heart was performed with an anti-human DAF MAb as first antibody (magnification 200x). Negative controls were performed using an isotype-matched irrelevant MAb (data not shown). (B) Immunostaining of DAF^{+/-} transgenic rat heart with human anti-Gal α 1-3Gal antibodies or an antihuman DAF MAb as first antibody followed by staining with a peroxidase-labeled antihuman or anti-mouse Ig, respectively (magnification 400x).

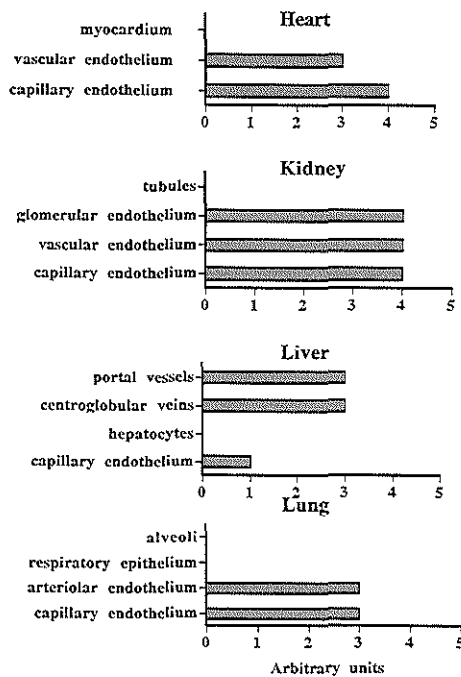


Figure 3. Semiquantitative analysis of DAF endothelial expression in tissues from transgenic line 52.2. DAF expression in endothelial cells from different tissues was determined by immunochemistry on cryosections as described in Materials and Methods. Results are expressed according to the intensity of specific staining (0= no staining; 1= low intensity; 2= medium; 3= high; 4=very high).

Levels of human DAF expressed by ECs isolated from transgenic rats

Endothelial cells from heart and lung were isolated from hemizygous ($DAF^{+/-}$) and homozygous ($DAF^{+/+}$) rats derived from founder 52.2 and from a nontransgenic rat as control. Endothelial cell populations were over 95% pure as assessed through an anti-rat ICAM-1 and anti-vWf antibodies. Cell surface expression of human DAF was investigated by flow cytometry following staining with an anti-DAF MAb. Figure 4 shows that the level of human DAF expressed on cardiac ECs from homozygous ($DAF^{+/+}$) rat was about 50% the level of DAF on human ECs (HUVEC). As expected, DAF expression was higher in homozygous ($DAF^{+/+}$) transgenic rats than in hemizygous ($DAF^{+/-}$) rats. Similar results were obtained with ECs isolated from lung (data not shown).

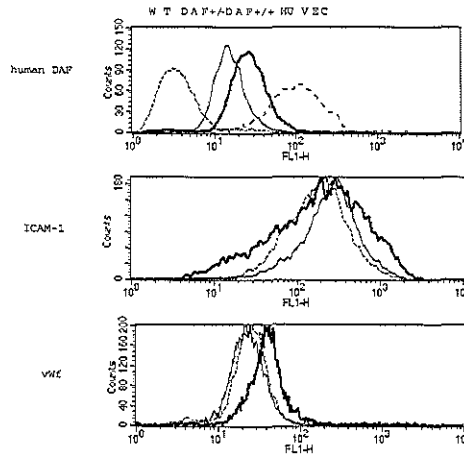


Figure 4. FACS analysis of human DAF expression by endothelial cells from hemizygous and homozygous transgenic rats. Endothelial cells were isolated from heart of wild type (WT) and transgenic rats, either hemizygous (DAF^{+/-}) or homozygous (DAF^{+/+}). Immunostaining was performed using an antihuman DAF MAb to compare levels of DAF expression on endothelial cells. An anti-rat ICAM-1 MAb and antihuman von Willebrand factor (vWf) antibody were used to assess the purity of endothelial cell preparations. Fluorescence was measured on 10,000 cells/sample using a FACS-calibur. Data are depicted in histograms plotting mean fluorescence intensity (MFI) on a log scale (x-axis) versus cell number (y-axis).

Protection of DAF-expressing rat ECs against primate antibodies and C-mediated cell lysis

The ability of human DAF to confer in vitro protection of rat ECs against primate xenogeneic (anti-rat) antibodies and complement-mediated cell lysis was investigated by incubating ECs isolated from transgenic rats with primate (cynomolgus) sera. Xenoreactivity of human serum for WT or transgenic rats EC was previously determined by flow cytometry analysis. Incubation of transgenic (hemizygous or homozygous) rat ECs with purified human anti-Gal α 1-3Gal antibodies showed binding of IgG and IgM XNA that was similar to that of ECs from WT nontransgenic rats (data not shown). Furthermore, ECs from either transgenic or control rats exhibited similar levels of Gal α 1-3Gal expression as measured by the binding of

FITC-labeled *Bandera simplicifolia* lectin (data not shown). In vitro protection of DAF-expressing rat ECs against antibodies and complement-mediated cell cytotoxicity was measured by incubating rat ECs with serial dilutions of primate (cynomolgus) serum. As shown in figure 5, primate serum induced a dose-dependent lysis of nontransgenic ECs for serum dilutions ranging between 6% and 25%. Compared to WT rat ECs, ECs isolated from transgenic rats were protected according to the level of human DAF expressed on the cell surface. When ECs were incubated in the presence of 25% serum, protection against cell lysis was $74 \pm 6\%$ ($P < 0.05$) and $43 \pm 4\%$ ($P < 0.05$) for homozygous and hemizygous, respectively, compared to WT rat ECs ($9.6 \pm 0.3\%$, $20.9 \pm 0.1\%$, and $36.8 \pm 0.1\%$ of cell lysis for homozygous, hemizygous, and WT, respectively).

Flow cytometry analysis of complement activation on ECs, following incubation with human serum, confirmed the ability of DAF expression to prevent C3 deposition on ECs since lower amounts of human C3 were detected on transgenic ECs than on WT ECs (figure 6). An overall reduction in C9 was also observed on rat ECs from homozygous transgenic rats whereas no effect of DAF expression was observed for C4 and factor B deposition.

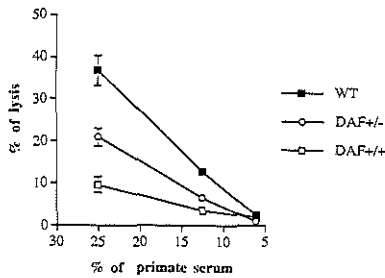


Figure 5. In vitro protection of ECs isolated from transgenic rats against primate xenogeneic antibodies and complement-mediated cell lysis. Cardiac endothelial cells from wild type (WT) and transgenic, either hemizygous (DAF^{+/-}) or homozygous (DAF^{+/+}), rats were labeled with Cr⁵¹ and incubated for 4 hr at 37°C with serial dilutions (from 25% to 6.12%, 50 μ l/well) of cynomolgus serum. Cell lysis was determined by measuring Cr⁵¹ release in culture supernatant as described in Materials and Methods. All experiments were done in triplicate, and the results are expressed as the mean of percent specific lysis \pm SD values. Results are representative of three independent experiments.

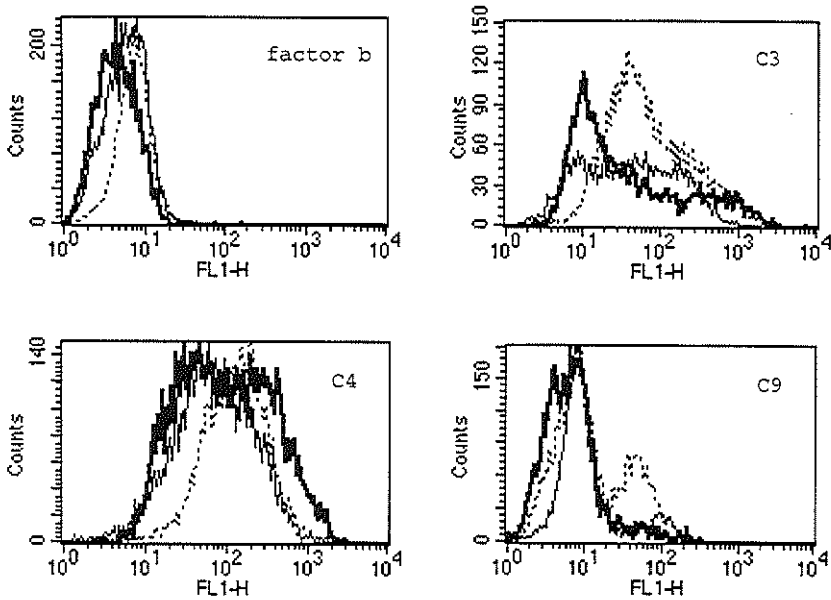


Figure 6. FACS analysis of in vitro human complement deposition on wild type versus transgenic rat ECs. Cardiac endothelial cells were isolated from wild type (dotted line) and transgenic, either DAF^{-/-} hemizygous (plain line) or DAF^{+/+} homozygous (bold line), rats. Immunostaining was performed on ECs incubated with human serum (dilution 1/10) for 30 min at 37°C. After incubation, ECs were washed and then incubated with either a FITC-labeled antihuman C3, C4, or factor B MAb or with an antihuman C5b-9 polyclonal rabbit serum followed by a FITC-labeled anti-rabbit Ab.

Ex vivo perfusion

The functional ability of hearts from hemizygous DAF^{-/-} rats to prevent complement activation was investigated by ex vivo organ perfusion with 15% human serum. Cardiac function, assessed by cardiac frequency, was monitored over a 60-min perfusion time (Figure 7). Our data showed that for DAF-expressing transgenic rats (n = 5), heart rate was not affected by serum perfusion and remained stable throughout the experiment. Similarly, perfusion of WT rat hearts with heat-inactivated serum (n = 5) showed normal cardiac function (data not shown). In contrast, cardiac function failed rapidly after perfusion of WT

rat hearts with XNA and complement-containing human serum and stopped at 11.2 ± 1.7 min ($n = 5$).

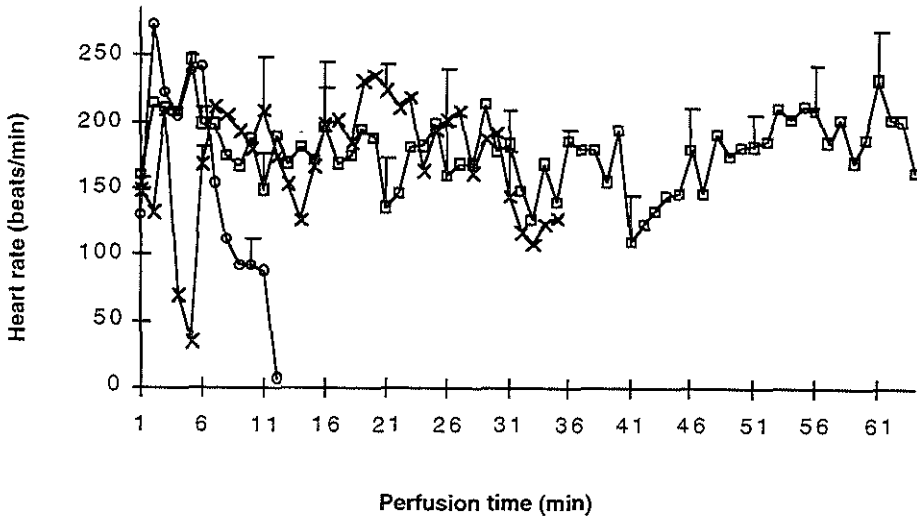


Figure 7. Cardiac function of transgenic rat hearts during ex vivo perfusion with human serum. Hearts from wild type (circles) ($n = 5$) or DAF^{+/-} hemizygous transgenic (squares) ($n = 5$) rats were perfused with 15% human serum for 60 min using a Langendorff apparatus. Heart rate (y-axis) was monitored every min (x-axis) throughout the experiment. Every point of analysis corresponds to the mean value of five experiments and SD is shown for values taken every 5 min.

Xenotransplantation of DAF⁺ transgenic rat hearts into primates

Transplantation of transgenic hearts was performed on cynomolgus to assess whether the expression levels of human DAF on ECs were sufficient to prevent HAR. We previously demonstrated that in this discordant combination, cardiac xenografts were hyperacutely rejected in 5.5 ± 1.4 min following binding of primate XNA and activation of complement through the classical pathway (19). To overcome a potential variability in xenoreactivity of primate recipients, hemizygous and homozygous transgenic hearts were grafted in each femoral vessel from the same recipient. Survival times of transplanted transgenic hearts are reported in Table 1. These results show that levels of DAF expression were correlated in vivo with the degree of protection against XNA and complement-mediated HAR. Nevertheless, protection for homozygous hearts compared to that for hemizygous hearts was higher than

expected from in vitro experiments (figures 5 and 6). Indeed, for hearts from hemizygous rats, rejection was delayed from 5 min to 15.5 min (n = 4) (P < 0.05 compared to WT), whereas cardiac xenograft survival was up to >4.5 hr for homozygous rats (n = 2) (P < 0.05 compared to WT or hemizygous transgenic hearts). Because the time of rejection could only be assessed on anesthetized primates, only a minimal estimation can be given here.

Histology of DAF^{+/-} rejected hearts, collected at the time of rejection, showed no significant evidence of edema or hemorrhage but showed microthrombosis, as previously described for nontransgenic rats (19). Nevertheless, differences were observed between tissue samples according to survival time. Indeed, a transplant which survived for 28 min, exhibited a significant PMN infiltration in the interstitium and binding to the endothelium of blood vessels compared to cardiac xenograft rejected at 11 min (figure 8). Biopsies from DAF homozygous heart transplants also showed an abundant leukocyte infiltration that was mostly composed of PMN, but also include a small fraction of monocytes. Vascular damage, focal necrotic lesions of myocardium, and interstitial hemorrhage were also observed.

Immunohistochemical analysis revealed deposition along endothelial surfaces of recipient IgG, IgM, and complement components of the classical but not the alternative pathway (Table 2). Less C3 and C9 fragment deposition was observed on transgenic tissues than on that of rejected organs from WT rats.

Table 1. Survival time of heterotopic cardiac xenografts of transgenic rat hearts (hemizygous DAF^{+/-} or homozygous DAF^{+/+}) transplanted into cynomolgus recipients.

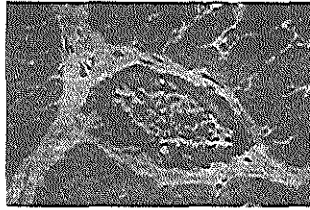
	Wild type rat	DAF ^{+/-}	DAF ^{+/+}
Survival time	5.5 ± 1.4 min	15.2 ± 7.4 min*	>1.5hr*** ^a
		(10, 11, 12, 28 min)	>4.5hr*** ^a
	n = 10	n = 4	n = 2

Rat hearts were grafted on femoral vessels bilaterally for hemizygous and homozygous rats. Wild-type heart survival has been previously reported (19).

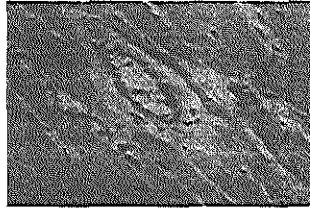
^aAnalysis of the last time points for each graft showing beating hearts (next analysis at 24 hr showed rejection).

*P < .05 for transplants from hemizygous DAF rats versus wild type rats.

**P < .05 for transplants from homozygous DAF rats versus wild type rats or hemizygous DAF rats.



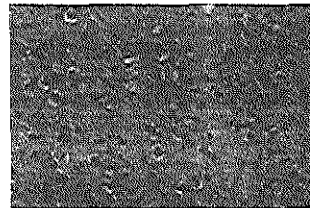
non-transgenic cardiac transplant rejected at 5 min



DAF^{+/-} cardiac transplant rejected at 11 min



DAF^{+/-} cardiac transplant rejected at 28 min



DAF^{+/+} cardiac transplant rejected at >1.5 hr

Figure 8. Histological analysis of DAF-expressing cardiac xenografts. Hearts were collected at the following times of rejection: 5 min for wild type, 11 min and 28 min for DAF^{+/-} transgenic rats, and after rejection for DAF^{+/+} transgenic rat. Tissue sections were counterstained with hematoxylin-eosin. (magnification 400x).

Table 2. Immunohistochemical analysis of cardiac xenografts for human Ig and complement deposition.

Rats	hDAF	vWf	h IgG	h IgM	C3	C4	C9	Fb
Ungrafted wild type (n=3)	-	++	-	-	-	-	-	-
Wild type (n=3)	-	+++	++	+	++	+ / +++	++	-
DAF ^{+/+} (n=3)	+++	+++	++	+	+	+	- / +	-
DAF ^{+/+} (n=2)	++++	+++	++	+	+	+	+ / -	-

Immunostaining of xenograft cryosections was performed as described in Materials and Methods. Results are expressed as a mean of experiments performed on two (DAF^{+/+} transgenics) or three (ungrafted, wild type, and DAF^{+/+} transgenic) animals. (-: no staining, +: low intensity, ++: medium intensity, +++: high intensity, ++++: very high intensity).

Discussion

Genetically engineered animals offer a unique opportunity for using donor animal organs for clinical transplantation. Transgenic pigs, which express molecules that could control immunological events associated with xenograft rejection, could be the first suitable source of these transplants. Transgenic mice are useful models for the analysis of promoter tissue specificity and transgene expression levels (24;25). However, there are no mouse models available for the study of transplantation of vascularized organs into primates. Investigation of transgenic or knockout mouse models (although restricted to ex vivo perfusion experiments) can be informative for several aspects of transgene function, but they do not allow analysis of the broad range of humoral and cellular interactions between graft and recipient that operate in vivo (25;26). Therefore, we have postulated that production of transgenic rats, which can be used for xenotransplantation into primates (19), could provide a useful in vivo model to test the benefits of genetic engineering interventions in xenotransplantation (22). The first step in validating this model was to demonstrate that endothelium-directed expression of human DAF in rat organs can delay xenograft HAR.

Transgene distribution in transgenic organs is a major concern for xenotransplantation. For membrane-bound proteins such as CRPs, transgenes should be expressed at least on ECs (the first target of XNA binding and complement activation), since few other cell types in the rat (and also the pig) express the Gal α 1-3Gal epitope (7). Our data show that endothelium-restricted transgene expression can be obtained in transgenic rats by using part of the human ICAM-2 gene promoter, and that tissue distribution of transgene expression in transgenic rats is almost identical to transgene expression in lines of transgenic mice (18).

The ability of human DAF to overcome primate XNA and complement-mediated rat cell lysis was demonstrated *in vitro* on isolated transgenic rat ECs. The data generated from this study provided *in vivo* confirmation of our previous *in vitro* experiments performed on transfected rat ECs, which demonstrated the ability of human DAF and CD59 to protect rat ECs from primate complement-mediated damage (20). Cardiac-derived ECs from rats homozygous for DAF expressed around 50% of the DAF levels observed in HUVECs. However, compared with other human cells, HUVECs express very high levels of DAF (3×10^5 molecules per cell compared with 8×10^4 molecules per cell for human leukocytes) (27;28). DAF levels on human ECs derived from organs have not yet been reported.

Because ECs from transgenic rats homozygous for DAF were more resistant to primate serum-mediated damage than ECs from rats hemizygous for DAF, *in vitro* inhibition of complement-dependent EC lysis and complement fragment deposition was said to be correlated with DAF levels. In *ex vivo* perfusion experiments, hemizygous DAF hearts were very efficiently protected from complement-mediated damage from 15% human serum, but showed only limited survival *in vivo*. Although a statistically significant improvement of graft survival time for hemizygous rats was observed (5 min for WT rats versus 15 min for hemizygous DAF rats), this has little relevance *in vivo*. The difference in *in vivo* versus *ex vivo* survival time is probably due to higher XNA and complement concentrations *in vivo* and the contribution of recipient blood cells absent from the *ex vivo* experiments. In contrast, the prolongation of xenograft survival to a minimal period of several hours (>1.5 hr to 4.5 hr) in homozygous DAF rats indicates that this level of transgene expression is sufficient to delay HAR in the absence of XNA depletion or immunosuppression of the recipient.

In some previous reports on the generation of transgenic animals for CRPs, levels of transgene expression have been quantified on ECs. Diamond et al. showed that expression of CD59 on aortic ECs from transgenic pigs reached 20% of the levels of HUVEC, conferred 50% protection against complement-mediated EC lysis *in vitro*, and compared with the controls, slightly prolonged xenograft survival (0.5 hr and 1 hr versus 2.2 hr and 3 hr, respectively) (29). Byrne et al. showed that aortic ECs expressed CD59 and DAF levels that were 67% and 45% of HUVEC levels, respectively, and had a 3- to 5-fold increased resistance against complement-mediated lysis *in vitro* (14). Xenografts were shown to have variable survival periods compared with controls (range 6 hr to 5 days versus 0.5 hr to 1 hr, respectively), and survival could be prolonged by depletion by depletion of xenoreactive antibodies (16). Importantly, in these transplantation trials, recipients also received extensive immunosuppressive and anti-coagulant treatment (14;16;29). Taken together these studies suggest that although moderate levels of DAF transgene expression (20-40% of HUVEC

levels) are able to confer protection against HAR in conditions in which complement or antibodies are limited (in vitro or ex vivo perfusion), a minimal threshold of protection in vivo requires DAF levels that are at least close to 50% of HUVEC levels. In agreement with this hypothesis, Cozzi et al. reported that transgenic hearts, which expressed DAF at levels several-fold higher than HUVECs (13), showed effective protection during ex vivo perfusion with human blood, and xenograft survival was considerably prolonged (5.1 days versus 1.6 days in controls) in the absence of any other treatment (15). However, these transgenic organs, even in immunosuppressed recipients, have been almost invariably rejected by AVR (30;31).

Nontransgenic rat hearts transplanted into primates have been rejected with a histological pattern of HAR showing endothelial cell damage, platelet microthrombi, and little PMN vascular accumulation (19). In contrast, rat hearts transplanted into primates treated with cobra venom factor (19) or DAF transgenic hearts that have survived transplantation for at least 28 min have been rejected, with a histological pattern of AVR showing extensive thrombosis, leukocyte accumulation (mainly PMN) in the vessels and tissues, edema, and hemorrhage. Furthermore, increased levels of DAF in transplanted organs (from homozygous and hemizygous DAF transgenic rats) result in longer periods of graft survival that correlate with progressive PMN infiltration, hemorrhage, and focal myocardial necrosis. Similarly, AVR is the most common result of xenotransplantation between discordant species (guinea pig-to-rat or pig-to-primate) after XNA depletion, complement inactivation of recipients, or transplantation of CRP transgenic organs (4;10;12;14-16;32;33). Although AVR in these models share common pathological features (hemorrhage, edema, and presence of leukocytes), they also show differences in the type of infiltrating leukocytes. In transplant combinations involving rats as recipients, mononuclear cell infiltrates predominate (33;34), whereas combinations involving primates as recipients (especially when the primates do not receive immunosuppressive drugs) show leukocyte infiltrates with mononuclear cells and a high proportion of PMN (4;15).

Taken together, our results demonstrate that a moderate level of human DAF expression on rat endothelium prolongs xenograft survival beyond HAR and that the PMN infiltration, hemorrhage, and focal myocardial necrosis associated with AVR in pig-to-primate transplants are also present in rat-to-primate transplants. It is likely that higher levels of DAF expression are required to further improve xenograft survival, or that other factors in addition to complement (such as XNA binding to ECs and mechanisms such as coagulation and EC activation) must be controlled to prevent xenograft AVR.

In conclusion, xenotransplantation of transgenic organs expressing CRPs, from either pig or

rat, prevents HAR but not the development of AVR, even when immunosuppressed primates are used as recipients (14-16;30;31). Therefore, there is an urgent need to express new transgenes to protect xenografts and decrease the side effects of immunosuppression. These transgenes should probably be aimed at decreasing Gal α 1-3Gal expression or to inhibiting coagulation and tissue infiltration by leukocytes. The development of transgenic rats represents a more rapid alternative than the production of transgenic pigs for testing the usefulness of new strategies and allows an easier analysis of mechanism of action. In this regard, the backcross of transgenic rats expressing DAF to prevent HAR, and the backcross of rats expressing Fas ligand in the endothelium (35) that could inhibit PMN adherence to endothelium (36) may facilitate analysis of the role of early leukocyte infiltration in AVR.

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CHAPTER 7

HUMAN DECAY ACCELERATING FACTOR EXPRESSED ON RAT HEARTS INHIBITS HUMAN LEUKOCYTE ADHESION

C.A.E. VERBAKEL, S. VAN DUIKEREN, R.L. MARQUET, J.N.M. IJZERMANS

LABORATORY FOR EXPERIMENTAL SURGERY, ERASMUS UNIVERSITY ROTTERDAM

submitted to Transplantation

Abstract 'Cellular immunity overrules the protective effect of hDAF as demonstrated in an ex vivo heart perfusion model' in Transplantation Proceedings 33(1-2): 781-782, 2001

Abstract

Background. In xenotransplantation the use of donors transgenic for recipient-type complement regulatory protein Decay Accelerating Factor (DAF/CD55) or Membrane Co-factor Protein (MCP/CD46) protects grafts against hyperacute rejection (HAR), which is primary mediated by xenoreactive natural antibodies (XNA) and complement (C). In the Langendorff model we have previously demonstrated that rat hearts transgenic for human CD55 (hCD55), perfused with human serum were protected against HAR. However, in the ex vivo situation these hearts were found to be destroyed by a process occurring after the period of HAR. We questioned whether transgenic hearts for hCD55 are also protected against adhesion and infiltration by cells implicated in the early phases of xenograft rejection. The aim of the present study was to analyse this process in the ex vivo heart perfusion model.

Methods. hCD55 transgenic rat hearts and their controls were perfused with either heat-inactivated or normal human blood solutions for 60 minutes. Although most of the hearts stopped beating within the 60 minutes perfusion period, the perfusion was not stopped to enable adhesion of cells during a fixed period identical for all groups.

Results. Independent of the presence of C, H&E-stained tissues of hCD55 transgenic hearts revealed less adhering PMN-leukocytes to the endothelium as compared to controls (mean: 31% versus 60%). Standard histology and immunohistochemistry showed that hCD55 transgenic hearts exhibited less interstitial edema, hemorrhage, microthrombosis, fibrin depositions and leukocyte infiltration as compared to controls. All hearts showed mild to moderate levels of P-selectin and similar levels of ICAM-1, C3c, C9, IgA, IgG and IgM depositions.

Conclusions. hCD55 expressed on rat hearts not only inhibits complement activation, but also human leukocyte-adhesion and apparently functions as an anti-adhesion molecule. hCD55 is not only an efficient factor in protecting grafts against HAR, but protects the graft against adhesion of leukocytes as well.

Introduction

Hyperacute rejection (HAR) is the first immunologic barrier to overcome, before organs can be transplanted between discordant species (1-3). HAR is primarily mediated by binding of xenoreactive natural antibodies to the vascular endothelium followed by complement activation, resulting in activation or damage of endothelial cells. The major features of HAR *in vivo* are platelet aggregation and adhesion, fibrin deposition, thrombosis, interstitial edema, hemorrhage and vasoconstriction. HAR can be averted by blocking either xenoreactive natural antibodies or complement (4;5). The second immunologic barrier in the discordant situation is acute vascular rejection (AVR), which can occur without the presence of T cells. AVR involves type II endothelial cell activation, including up-regulation of proinflammatory molecules, infiltration of host monocytes and natural killer cells into the graft, and cytokine production, leading to chemotaxis of leukocytes (4). The third and probably most difficult immunologic barrier to overcome is the cellular immune response, in which similar mechanisms as in allograft rejection are involved. The use of donors transgenic for a certain recipient-type complement regulatory protein protects grafts against hyperacute rejection (HAR). We demonstrated earlier that rat hearts transgenic for human CD55 (hCD55), perfused with human serum, were protected against HAR (6). Although the hearts were not rejected by HAR, they were finally rejected by a delayed form of HAR. Since CD55 prolongs the survival of hearts perfused with human serum by regulating the activation of endothelial cells, we wondered whether CD55 might have an extra effect, i.e. inhibiting the adhesion of cells to the vascular endothelium. Others have found that CD55 indeed can function as an anti-adhesion molecule (7). Therefore, the aim of our study was to analyze whether rat hearts transgenic for hCD55 are protected against cell-mediated immunity upon perfusion with human blood in the Langendorff device. The contribution of complement in the adhesion process was analyzed by using either normal or de complemented (=heat-inactivated) blood.

Materials and Methods

Animals

Rats

Male and female heterozygous and homozygous hCD55 transgenic rats were bred by using a construct containing the hCD55 cDNA under the transcriptional control of the endothelial cell-specific human ICAM-2 promoter. hCD55 expression was evaluated by immunohistology, FACS analysis of purified endothelial cells, and Southern blot techniques as described by Charreau et al. (6). Wistar rats were used as controls. When control hearts were perfused with

12% control blood solution, the mean beating time was 55 minutes, and if pure Krebs-Henseleit solution was used, hearts kept on beating for 190 minutes. Based on the results in the controls, we performed a 60 minutes perfusion of all hearts in the experimental groups. The animals were housed in a certified animal breeding facility under standard conditions and had free access to standard rat chow and water acidified to pH 2.2 ad libitum. The experimental protocol was approved by the committee on Animal Research of the Erasmus University.

Surgical procedure and ex vivo heart perfusion (Langendorff)

Prior to the surgical procedure, rats were anesthetized with isoflurane inhalation and 1.0 mL Heparin (50 IU/mL) was injected intravenously. Via two lateral incisions the thorax was opened and the heart was removed. A 14-gauge in-dwelling cannula was inserted into the aorta and secured with a 4.0 ligature. The heart was contiguously perfused with oxygenated Krebs-Henseleit (KH) solution using a syringe and directly linked to the Langendorff circuit, as previously described by Verbakel et al. (8). All hearts were perfused with KH solution (pure KH solution, without Heamaccel) for 10 minutes and were then switched to perfusion for 60 minutes with 12% human blood or 12% heat-inactivated human blood. During this 60 minutes perfusion, hearts were monitored constantly for apex frequency and flow using multichannel registration. Most of the hearts perfused with blood stopped beating within the 60 minutes perfusion period. The perfusion was not stopped, to enable adhesion of cells during a fixed period identical for all groups. The time measured until they stopped beating was also noted.

Perfusion solutions

Frozen, pooled, human O-type plasma was obtained from the blood bank of the University Hospital Dijkzigt (Rotterdam, The Netherlands). For each experiment fresh human serum was prepared from thawed human plasma after the induction of clotting with Thrombin (0.5 mL Thrombin / 200mL human plasma). Heat-inactivation, ie complement inactivation, was realized by heating of the serum at 56° for 30 minutes. O-type blood was collected in 9-mL EDTA test tubes and immediately processed as follows. After centrifugation (2000 rpm, 10 minutes), the plasma was removed and replaced by a similar volume of serum. To prepare the 12% (heat-inactivated) solution for perfusion, the blood was diluted with KH stock solution, prepared as described earlier. In the blood solutions erythrocytes, platelets and white blood cells (WBC) were present. A sample of the 12% (heat-inactivated) human blood mixture was taken from each fresh prepared solution and the WBC count was performed in a blood cell

counter (Sysmex F800, Japan). The mean WBC concentration was $0.5 \times 10^9/L$. In all perfusion solutions we finally added 0.1-0.2 mL heparin (50 IU/mL) to prevent coagulation.

Immunohistochemistry

After 60 minutes perfusion each heart was cut in two. One sample was fixed in 3.6% formaldehyde solution. Following dehydration and paraffin embedding, 4 μm -thick sections were cut and stained for Hematoxylin & Eosin (H&E). By means of standard histology, all hearts were analyzed for differences in amounts of leukocytes adhering to the endothelium. In these stained tissues twenty medium sized vessels were examined to evaluate the presence of leukocytes. In each vessel the amount of leukocytes adhering to the endothelium and the amount of leukocytes present in the lumen were counted. Finally, the percentage adherence was calculated. The other part of the sample was snap frozen and stored at -80°C until sectioned for immunohistochemistry. Frozen tissue sections (6 μm -thick) were prepared in a Microm HM 560 cryostat and stained for rabbit antihuman C3c complement Fluorescein-Iso-Thio-Cyanate (FITC), rabbit antihuman C9-antibodies, mouse antirat CD54 (=ICAM-1), rabbit antihuman CD62P (=P-selectin), and rabbit antihuman IgA, IgG, IgM. To stain rat P-selectin, rabbit antihuman CD62P was used, since this crossreacts with rat P-selectin. C3c and C9 staining was done as previously described by Verbakel (8). P-selectin, (1:200), IgA (1:400), IgG (1:400) and IgM (1:400) staining were performed in the same way as C9-staining (12). ICAM-1 stainings were performed in the following way: the frozen sections were thawed at room temperature for 1 hour, fixed with acetone for 10 minutes and air dried for 15 minutes. With a Peroxidase Anti Peroxidase(PAP)-pen circles were drawn around the tissue sections and washed with PBS three times. Sections were incubated for 10 minutes with 0.03-0.1% H_2O_2 in humidified atmosphere and rinsed three times with PBS. After preincubation for 15 minutes with 0.1% normal rabbit serum, diluted (1:200) mouse antirat ICAM-1 antibody was added and the sections were incubated for 60 minutes in humidified surroundings. Again sections were washed three times with PBS. Sections were incubated with secondary rabbit anti mouse antibody for 30 minutes and washed three times with PBS. Diaminobenzidine (DAB) was added and sections were incubated for 5-15 minutes. The DAB-reaction was stopped by flushing with water for 5 minutes. Sections were put in Mayer's haematoxylin for 40 seconds, washed, and were then put in an ammonia-solution. Dehydration was followed by a Xylene step. The slides were covered with a coverslip.

Antihuman C3c-stained sections were examined under a fluorescence microscope, and antihuman C9-, P-selectin-, ICAM-1-, IgA-, IgG- and IgM-stained sections under the light microscope. The intensity of C3c- and C9-depositions, and P-selectin and ICAM-1-expression

on the heart tissues was scored blindly via semiquantitative analysis: 0 = no staining, 0.5 = mild staining, 1 = dense staining. IgA, IgG and IgM depositions were scored semiquantitatively as well: 0= no staining, 0.5 = mild staining, 1 = moderate staining, 2 = dense.

The experimental groups

Group 1: Control hearts perfused with 12% human blood (n=6)

Group 2: hCD55 hearts perfused with 12% human blood (n=11)

Group 3: Control hearts perfused with 12% heat-inactivated human blood (n=8)

Group 4: hCD55 hearts perfused with 12% heat-inactivated human blood (n=8)

Statistical Analysis

The non-parametric Kruskal-Wallis test was done to see what test had to be used. The non-parametric Mann-Whitney test was performed on (1) the difference in cell adhesion in H&E stained tissues, and (2) P-selectin expression. The Student's *t* test was used to evaluate (1) xenograft survival, (2) C3c deposition, (3) C9 deposition, (4) ICAM-1 expression, (5) IgA, IgG and IgM depositions. All tests were considered significant when $P < .05$.

Results

Langendorff perfusions

The mean beating times (MBTs \pm SD) for all groups are shown in table 1. Control hearts perfused with human blood (group 1) beated significantly shorter than group 2, i.e. hCD55 hearts perfused with human blood ($P = .008$), and group 4, i.e. hCD55 perfused with heat-inactivated ($P = .045$). Similar beating times were found in control hearts and hearts transgenic for hCD55 perfused with heat-inactivated human blood (group 3 and 4).

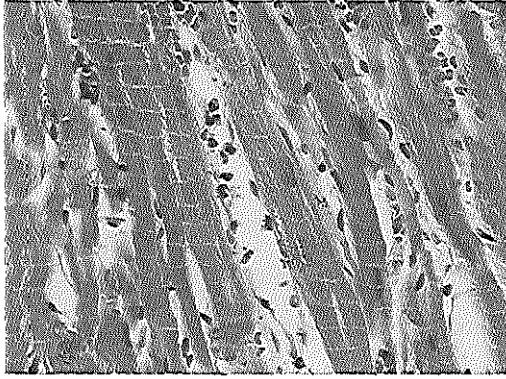
Table 1. Mean beating times (MBTs) of hearts from hCD55 transgenic rats and controls perfused with either human blood (HB) or heat-inactivated human blood (HIHB)

Group	MBT (min) \pm SD
1. controls + HB	19 \pm 11
2. hCD55 + HB	43 \pm 19
3. controls + HIHB	34 \pm 15
4. hCD55 + HIHB	38 \pm 19

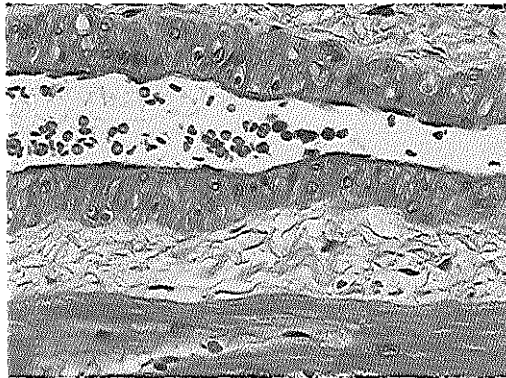
$P < .05$: 1vs2

Histology

Standard histology showed interstitial edema, hemorrhage, fibrin depositions, microthrombi and cellular infiltration. The cellular infiltrates contained leukocytes, being predominantly polymorphonuclear cells (PMN) (figure 1).



A



B

Figure 1. Standard histology showed interstitial edema, hemorrhage, fibrin depositions and microthrombi. These features were more prominent in control hearts (A) than in hCD55 transgenic hearts (B). Cellular infiltrates contained leukocytes, being predominantly polymorphonuclear cells (PMN). In controls (A) the percentage PMN adhesion is significantly higher than in hCD55 hearts (B).

Human leukocyte adhesion to endothelial cells

The percentage leukocytes adhering to the vessel wall in rat hearts transgenic for hCD55 and control hearts, perfused with human blood are summarized in Table 2. In H&E-stained hCD55 hearts perfused with human blood only 34% adherence of leukocytes was found, whereas in control hearts perfused with human blood 66% of the leukocytes adhered ($P = .003$). H&E-stained hCD55 hearts perfused with heat-inactivated human blood (group 4) showed significantly fewer adhering leukocytes than control hearts perfused with heat-inactivated human blood (group 3; $P = .002$).

Table 2. Percentage of leukocytes adhering to the vessel wall \pm standard deviation (SD) in hCD55 transgenic rat hearts and controls perfused with 12% human blood (HB) or heat-inactivated human blood (HIHB) in the Langendorff system.

Group	Percentage of adhering Leukocytes (H&E) \pm SD
1. controls + HB	66 \pm 14
2. hCD55 + HB	34 \pm 12
3. controls + HIHB	53 \pm 14
4. hCD55 + HIHB	28 \pm 9

$P < .05$: 1vs2, 3vs4

Adhesion molecule expression

An overview of the mean adhesion molecule expression per experimental group (\pm SD) is given in Table 3. hCD55 transgenic hearts perfused with human blood (group 2) expressed no P-selectin on their vascular endothelium. This differed significantly from all the other groups ($P < .003$). Controls perfused with human blood (group 1), controls perfused with heat-inactivated human blood (group 3), and hCD55 hearts perfused with heat-inactivated human blood (group 4) expressed similar levels of P-selectin. The ICAM-1 expression was found to be similar in all experimental groups (data not shown).

Table 3. Mean score \pm standard deviation (SD) of P-selectin, C3c, C9, IgA, IgG and IgM deposition in rat hearts perfused with human blood (HB) or heat-inactivated human blood (HIHB)

Group	P-selectin	C3c	C9	IgA	IgG	IgM
1. controls + HB	1.2 \pm 1.0	0.8 \pm 0.4	0.5 \pm 0.4	1.5 \pm 0.7	0.7 \pm 0.3	1.5 \pm 0.7
2. hCD55 + HB	0	0.3 \pm 0.5	0.2 \pm 0.3	0.7 \pm 0.4	0.8 \pm 0.4	0.8 \pm 0.5
3. controls + HIHB	1.1 \pm 0.6	0.4 \pm 0.4	0.4 \pm 0.4	0.8 \pm 0.3	0.8 \pm 0.5	1.1 \pm 0.6
4. hCD55 + HIHB	1.5 \pm 0.1	0.4 \pm 0.4	0.4 \pm 0.4	0.9 \pm 0.5	0.9 \pm 0.2	1.0 \pm 0.7

$P < .05$: P-selectin: 1vs2, 2vs3, 2vs4

Anti-human C3c and C9 depositions

An overview of the means of antihuman C3c and C9 depositions per experimental group (\pm SD) are given in Table 3. C3c and C9 were mostly present diffuse along the cardiomyocytes and on some vessels. Both control groups expressed similar levels of C3c and C9 as compared to the hCD55 transgenes. A trend is seen in the pattern of C3c and C9 depositions: control hearts perfused with human blood (group 1) expressed higher levels of C3c and C9 in contrast to the other experimental groups, although these differences were not significant.

IgA, IgG, IgM depositions

An overview of the means of antihuman IgA, IgG, IgM deposition per experimental group (\pm SD) are given in Table 3. Control and hCD55 transgenic hearts perfused with human blood exhibited IgA, IgG and IgM not only on the endothelium, but diffuse on the cardiomyocytes as well. In group 1, i.e. control hearts perfused with human blood, IgA and IgM reached high levels, whereas IgG did not, but these differences were not significant. In hCD55 transgenes perfused with human blood (group 2) all immunoglobulin levels were found to be similar. Control and hCD55 hearts perfused with heat-inactivated human blood (group 3 and 4) expressed the same levels of IgA, IgG and IgM.

Discussion

In discordant xenotransplantation both natural antibodies and complement are crucial factors of inducing HAR. Since HAR has almost been solved by using donors transgenic for recipient-type complement regulatory proteins, it is the second immunologic barrier which has to be overcome: the acute vascular rejection (AVR) process. During AVR type II endothelial cell activation plays a central role via up-regulation of proinflammatory genes in endothelial cells and cytokine production (3;4;9-11). Thus promoting the adherence of leukocytes.

The aim of our study was to analyse the intermediate phase between HAR and AVR in *ex vivo* perfused rat hearts, the so called delayed HAR phase. hCD55 transgenic rat hearts and controls were perfused with different blood solutions and adhering leukocytes were counted. If we consider the control group perfused with human blood, 66% leukocyte adherence was observed, which correlated with a moderate and mild expression of P-selectin and ICAM-1, respectively. Antihuman C3c and C9 depositions were present on the vascular endothelium and along the cardiomyocytes. The most important finding of the present study was that hCD55 hearts perfused with human blood showed low levels of C3c and C9 depositions, a moderate expression of ICAM-1 and no P-selectin, and fewer leukocytes adhering to the endothelial cells (34%) as compared to controls. The control group perfused with heat-inactivated human blood, showed a similar leukocyte adhesion percentage as the hearts perfused with normal blood, indicating that not the absence of complement, but hCD55 is responsible for the inhibition of adherence. hCD55 hearts perfused with heat-inactivated blood, showed low levels of C3c and C9 depositions and, as expected, low numbers of adhering leukocytes (28%). The adhering cells were predominantly polymorphonuclear cells (PMN). Adhesion of human PMN to porcine aortic endothelial cells and inhibition of adhesion by hCD55 has been demonstrated earlier (12). Our findings suggest that hearts expressing hCD55 might be protected against PMN-mediated rejection. Moreover, in previous experiments we found that rat hearts expressing hCD55 transplanted into primates showed a PMN-dominated infiltrate upon rejection (6).

Our hypothesis was that when a delay in the activation of endothelium occurred, this would lead to a decline in the number of leukocyte-endothelium interactions and a low expression of P-selectin. This was only found to be true for the hCD55 hearts perfused with human blood (group 2). In the heart perfused with heat-inactivated human blood (group 3 and 4), we found significantly less leukocyte-endothelial cell interactions in the hCD55 transgenic hearts, but the level of P-selectin expression was as high as in controls. We may therefore tentatively conclude that hCD55 acts as an anti-adhesion molecule, independent of the presence of

complement. The beneficial effect of a high expression of hCD55 in the donor is not only restricted to HAR, but also the ensuing process of AVR.

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CHAPTER 8

NEGLECTIBLE ROLE FOR NK CELLS AND MACROPHAGES IN DELAYED XENOGRAFT REJECTION

K.A. VAN OVERDAM¹, C.A.E. VERBAKEL¹, E.A. KOUWENHOVEN¹,
N. VAN ROOIJEN², R.W.F. DE BRUIN¹, J.N.M. IJZERMANS¹, R.L. MARQUET¹

¹LABORATORY FOR EXPERIMENTAL SURGERY, ERASMUS UNIVERSITY ROTTERDAM

²DEPARTMENT OF HISTOLOGY, FREE UNIVERSITY OF AMSTERDAM

Abstract

Hyperacute rejection (HAR) of a discordant xenograft can be avoided by complement manipulation, but delayed xenograft rejection (DXR) still leads to graft loss. It is generally assumed that macrophages and NK cells play key roles in DXR. In the present study the survival times and cellular infiltrate following guinea pig to rat heart transplantation was analyzed in the course of DXR, following aspecific and specific manipulation of macrophages and NK cells. HAR was overcome by a single injection of cobra venom factor 1 day before heart transplantation. To aspecifically reduce the inflammatory response dominating DXR, dexamethasone (DEXA) was given. Treatment with DEXA markedly reduced infiltration by NK cells, macrophages, and granulocytes. It also led to prolonged graft survival times (median survival of 0.4 days, $n = 10$, $P < 0.05$). In the second series of experiments the specific roles of NK cells and macrophages in DXR were further assessed. Monoclonal antibody 3.2.3 was used to selectively deplete NK cells. Liposome-encapsulated dichloromethylene-biphosphonate was given to achieve macrophage depletion. Neither of these specific treatments, alone or combined, led to prolonged graft survival. Immunohistology revealed that at day 2 after transplantation no NK cells or macrophages were present in grafts from the combined treatment group. Only a mild infiltration of granulocytes was observed. Collectively, these results strongly suggest that NK cells and macrophages are not likely to be pivotal cell types in DXR.

Introduction

If a recipient of a discordant xenograft is treated appropriately to avoid hyperacute rejection (HAR), delayed xenograft rejection (DXR) occurs after a few days through as yet undefined mechanisms. In the guinea pig (GP) to rat combination, administration of cobra venom factor (CVF) will overcome HAR of GP hearts, which then will survive for up to 3 days (1). DXR in this complement-depleted model is characterized by a prominent cellular infiltration and endothelial deposition of xenoreactive natural antibodies (2). It has been shown that DXR involves a T cell-independent infiltration of macrophages and NK cells, in association with a marked expression of proinflammatory cytokines (3).

The aim of the present study was to investigate whether two different approaches to inhibit typical DXR-related infiltration, might prolong the survival of hearts in the GP to rat model. The aspecific approach consisted of treatment of recipients with dexamethasone (DEXA), a drug known for its capacity to inhibit inflammation in its broadest sense. In the more specific approach, NK cells and macrophages were selectively depleted by the following methods. Monoclonal antibody (mAb) 3.2.3 was used to deplete NK cells. This mAb recognizes a unique triggering structure present on fresh and Il-2-activated NK cells (4). Previous studies revealed that intraperitoneal treatment of rats with mAb 3.2.3 for 3 days can completely and selectively eliminate NK activity in the spleen and peripheral blood for at least 10 days (5). To assess the role of macrophages, we eliminated macrophage function using liposome-encapsulated dichloromethylene-biphosphonate (Cl₂MBP). On injection, these liposomes are rapidly phagocytosed by macrophages, leading to intracellular release of Cl₂MBP which eventually kills the cell. Cl₂MBP has been demonstrated to eliminate macrophages for about 7 days after intravenous injection (6;7).

Materials and Methods

Animals

Animals were obtained from Harlan-CPB (Austerlitz, The Netherlands). Male inbred Lewis rats were used as recipients and female Dunkin Hartley GPs as donors, GPs and rats weighed approximately 250 g when used. The experimental protocol was approved by the Committee on Animal Research of Erasmus University.

Heart transplantation

GP hearts were transplanted heterotopically into rats according to the technique described by Ono and Lindsey (8). Graft function was assessed by daily palpation. End of graft survival was defined as the day on which palpation indicated total loss of contractile activity, this was confirmed by inspection at laparotomy.

Complement depletion

To deplete recipients of complement CVF was used (*Naja naja kaouthia*). CVF purification was performed according to Beukelman et al. (9). A single dose of 0.1 ml CVF (with a complement-depleting activity of 30 000 U/ml) dissolved in 0.9 ml PBS was administered i.v., 1 day prior to heart transplantation.

Dexamethasone

DEXA was reconstituted in PBS and injected i.m., daily in a dose of 0.5 mg/kg, starting 1 day before transplantation.

Graft infiltrating cells

Immunohistological analysis of grafts undergoing DXR was performed at different intervals after transplantation, as described previously (10). Immunoperoxidase staining on frozen sections was done using the following monoclonal antibodies:

ED-1, W3/25, OX8, 3.2.3 (courtesy of Dr. Eggermont DDHK, Rotterdam, The Netherlands) and HIS48 to demonstrate monocytes/macrophages, CD4⁺ T cells, CD8⁺ T cells, NK cells, and granulocytes, respectively. The amount of infiltrating cells was scored semiquantitatively (from – to + + + +).

Depletion of NK cells

Selective NK cell depletion was obtained by intraperitoneal administration of 0.1 ml purified mAb 3.2.3 (0.5 mg/ml) dissolved in 0.9 ml PBS on days –1, 0 and 1.

Depletion of Macrophages

Liposome-encapsulated Cl₂MBP was used for selective depletion of macrophages. Liposomes were prepared as described previously (6). Rats received 0.5 ml Cl₂MBP-liposomes suspension (5 mg Cl₂MBP/ml) i.v. on days –4 and –1.

Study design

Rats were divided into the following groups: group 1 (n = 19): no treatment, group 2 (n = 19): complement depletion by CVF, group 3 (n = 10): treatment with CVF and DEXA, group 4 (n = 4): complement depletion by CVF and NK cell depletion by 3.2.3., group 5 (n = 5): complement depletion by CVF and macrophage depletion by liposomes, and group 6 (n = 9): treatments as in groups 4 and 5 combined. Analysis of infiltrating cells was done on day 2 after transplantation on grafts from animals treated as in groups 2, 3 and 6, using 3 animals per group. Day 2 was chosen as the best representative for the presence of DXR (see Table 1).

Statistical analysis

Student's *t*-test was used to evaluate the differences (considered significant when $P < 0.05$) between group mean values. Survival times are given as median survival time (MdST) and range.

Results

Survival times

The survival times obtained in groups 1-6 are given in Table 1. Control GP grafts in group 1 had a MdST of 16 min. (range 8-55 min.). Treatment with CVF resulted in a MdST of 2 days (range 1-4 days). Addition of DEXA (group 3), not only led to a significantly prolonged MdST of 4 days (range 2-5 days) but also to an improved performance of the heart grafts. The hearts showed less edema and hemorrhage and kept on beating vigorously until 1 day before rejection. This compares favorably with hearts from group 2, which performed poorly from day 1 onwards, exhibiting extreme hemorrhagic edema. Depletion of NK cells (group 4) or macrophages (group 5) in recipients treated with CVF had no effect on graft survival time. The MdSTs were 2 days, similar to those following treatment with CVF alone. Also the combined treatment (group 6) did not lead to prolonged graft survival.

Graft infiltrating cells

The results are summarized in Table 2. In animals treated with CVF only, graft infiltrating cells mainly consisted of NK cells, macrophages, and granulocytes. Only a few T cells were demonstrable. Hearts from animals treated with CVF and DEXA contained no T-cells, NK cells, and macrophages and only a few granulocytes. Heart grafts from group 6, treated with 3.2.3 and macrophage-depleting liposomes, showed a similar picture, no T cells, NK cells, or macrophages but only a mild infiltration of granulocytes.

Table 1. Graft survival times in the guinea pig-to-Lewis rat combination

Group / treatment	MdST	Range	N
1 none	16 minutes	8-55	19
2 CVF	2 days	1-4	19
3 CVF and DEXA	4 days	2-5	10
4 CVF and 3.2.3	2 days	2-3	4
5 CVF and liposomes	2 days	2-3	5
6 CVF and 3.2.3 plus liposomes	3 days	1-3	9

MdST: median survival time. CVF: cobra venom factor. DEXA: dexamethasone 0.5 mg/kg/day. Group 2 vs group 3: P<0.05. Monoclonal antibody 3.2.3. to deplete NK cells: 0.1 ml on days -1, 0 and 1. Liposome-encapsulated Cl₂MBP to deplete macrophages: 0.5 ml on days -4 and -1.

Table 2. Graft-infiltrating cells on day two following guinea pig-to-Lewis rat heart transplantation

Group / treatment	T	NK	Mo / Ma	Gran
2 CVF	∇	++++	+++	+++
3 CVF and DEXA	-	-	-	∇
6 CVF and 3.2.3 plus liposomes	-	-	-	+

Treatment: see Table 1. Infiltrating cells: given as mean scores, n=3 / group. T-cells: CD4⁺ and / or CD8⁺ cells. NK: 3.2.3. positive cells. Mo / Ma: ED-1 positive cells. Gran: HIS48 positive cells.

Discussion

It has been demonstrated repeatedly that when HAR of a discordant xenograft is blocked by preventing the activation of complement, grafts are still rejected after a few days (1;2;11). The mechanism underlying this rejection process, called DXR, is still not clear. Immunohistological examination of grafts undergoing DXR has revealed that macrophages and NK cells are abundantly present, which suggests that these cells participate in the rejection process. Our present results confirm that grafts subjected to DXR are heavily infiltrated by macrophages and NK cells. From earlier studies we know that NK cells are already demonstrable from the day of transplantation onward, whereas macrophages start

infiltrating from day 1 (12). The assumption that the presence of macrophages and NK cells would imply their actual involvement in DXR sounds logical but is not supported by our current results. Firstly, when DEXA was given to CVF-treated recipients virtually no infiltrating cells could be demonstrated. Such grafts survived significantly longer than CVF-treated controls, but still were rejected within an additional 2 days. The major gain of DEXA treatment was that the hearts performed much better because edematous enlargement of the grafts was postponed. It is known that DEXA is capable of inhibiting various aspects of the inflammatory response, including production of Il-6 and TNF α (13;14). In earlier experiments we showed that DEXA had a profound inhibitory effect on the early TNF α response, which suggests that the early hemorrhagic deterioration of a graft during DXR may be mediated by this cytokine (11). The second line of evidence that macrophages and NK cells are innocent bystanders rather than the actual executors of DXR comes from our results obtained with NK cell and macrophage depletion. Neither the removal of NK cells nor the depletion of macrophages had any effect. Also, when both treatments were combined, no effect on survival was observed. In addition, immunohistochemical analysis of grafts from the latter group demonstrated the efficacy of the treatment, since no NK cells and macrophages were found to be present. Several other investigators have tried to define *in vivo* the effector cells in DXR. Fryer et al. (15) used similar macrophage-depleting liposomes, as we did in the present experiments, and also failed to improve discordant xenograft survival in two different GP to rat models. From our studies we conclude that DXR in the rather harsh GP to rat model is not likely to be mediated by macrophages and NK cells.

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CHAPTER 9

UPREGULATION OF HEME OXYGENASE-1 (HO-1) PROTECTS hCD55 TRANSGENIC HEARTS FROM ISCHEMIC INJURY, BUT DOES NOT PROLONG XENOGRAFT SURVIVAL

C.A.E. VERBAKEL¹, R.E. BEEKHUIS¹, S. VAN DUIKEREN¹, S. DEKKER²,
R.W.F. DE BRUIN¹, R.L. MARQUET¹, J.N.M. IJZERMANS¹

¹LABORATORY FOR EXPERIMENTAL SURGERY, ERASMUS UNIVERSITY ROTTERDAM

²DEPARTMENT OF CELL BIOLOGY & GENETICS, ERASMUS UNIVERSITY ROTTERDAM

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Abstract

Heme oxygenase-1 (HO-1) is a molecule known to diminish ischemia/reperfusion (I/R) injury. In xenotransplantation, ischemia reperfusion injury may play a pivotal role in graft dysfunction and organ loss. The aim of the present study was to determine whether upregulation of HO-1 would lead to improvement of the performance of mouse hearts perfused with human serum in the Langendorff circuit. Upregulation of HO-1 was realized by intraperitoneal administration of Cobalt Protoporphyrin IX (CoPP), and confirmed by Northern blot analysis. Control animals were injected with NaCl. We first investigated the effect of HO-1 upregulation alone on the performance of non-transgenic mouse hearts. To evaluate the recovery of myocardial function, non-transgenic hearts, pretreated with either NaCl or CoPP, were perfused with Krebs-Henseleit (KH) solution for 10 minutes, after which 30 minutes of ischemia was given, followed by 45 minutes of reperfusion with KH. Secondly, we analyzed the effect of HO-1 upregulation on the performance of hCD55 (hDAF) transgenic mouse hearts and their controls by perfusing with human serum. The infarct size was measured by staining of the hearts with tetrazoliumchloride (TTC). A significant difference in the recovery of myocardial function was detected between control mice (group 1), in which 6 out of 13 hearts were unable to regain contractions, and HO-1 induced mice (group 2), in which 3 out of 12 hearts were unable to regain contractions. The infarct size and the recovery of the flow were also significantly different, in advantage of the HO-1 induced groups. Mouse hearts transgenic for hCD55 survived significantly longer than controls, in both CoPP and NaCl injected groups. In addition, a reduction of the infarct size was found in the HO-1 induced transgenic group, as compared to their controls. Our findings indicate that upregulation of HO-1 protects hearts from ischemic injury by reducing the infarct size. Furthermore, HO-1 improves the performance of the hearts, however, an additional effect of HO-1 on the prolongation of xenograft survival could not be detected.

Introduction

One approach to overcome the shortage of donor organs is the use of animals as a source of organs for transplantation, i.e. xenotransplantation. Xenotransplantation is defined as the transplantation of cells, tissues or organs between different species. Progress has been made in clarifying some of the barriers to xenotransplantation and defining appropriate therapeutic interventions aimed at the removal of xenogeneic natural antibodies and at the limitation of complement activation, both responsible for hyperacute rejection (HAR) (1). Apart from the immunological mechanisms, I/R injury may play a role in xenotransplantation, leading to early graft dysfunction. The exact mechanism remains unclear, but data suggest that oxidative stress and inflammatory responses play a major role. Reperfusion in itself is believed to bring about cellular injury. Oxygen free radicals and other toxic metabolites are formed when oxygen and energy supply balances are restored during reperfusion. One of the physiological protection mechanisms against the production of oxygen free radicals is upregulation of heme oxygenase. In vivo, heme oxygenase activity is upregulated by various stimuli, i.e. hypoxia, oxidized haemoglobin, oxygen free radicals, heavy metals, hydrogen peroxide or metalloporphyrins, and it is considered one of the most sensitive indicators of cellular stress (2;3). There are three isoforms of heme oxygenase (HO). HO-1, also known as heat shock protein 32 (HSP32), is the rate-limiting stress enzyme, which catalyses the degradation of heme into equimolar amounts of biliverdin, carbon monoxide (CO) and free iron. HO-2 catalyzes the same reaction as HO-1, but it differs in many respects and is regulated under separate mechanisms (4;5). HO-3 which is expressed constitutively, has very low activity and its function probably involves heme binding (6). In analogy with heat shock regulation, upregulation of HO-1 may be an adaptive mechanism protecting cells from stress, in particular ischemia. The upregulation of HO-1 has been shown to be cytoprotective and also reduces immune responses, i.e. decrease of IFN γ and IL-2 production (2;7-9). Several mechanisms have been postulated for the cytoprotective actions of HO-1. The elimination of pro-oxidant free heme could decrease the formation of hydroxyl radicals. HO-1 mediates the production of free radical scavengers with antioxidant properties. Carbon monoxide (CO) would act as vasodilator and inhibits platelet aggregation. "Free" iron increases oxidative stress and upregulates the cytoprotective gene, ferritin (9-12).

In our experiments, upregulation of HO-1 activity was accomplished by administration of Cobalt Protoporphyrin IX (CoPP). This metalloporphyrin is believed to have the capacity to produce an oxidant stress by generating or increasing the intracellular content of active oxygen species, thereby stimulating the expression of HO-1 (2;7;13). The aim of the study was first to examine whether CoPP in our mouse model was able to bring about an

upregulation of HO-1. Secondly, we examined the effect of HO-1 upregulation on the performance of mouse hearts perfused in the Langendorff device. Subsequently, the effect of HO-1 upregulation was examined in hCD55 transgenic mouse hearts perfused with human serum.

Materials and Methods

Animals

Male and female heterozygous and homozygous B₁₀CBA-mice transgenic for hCD55 (hDAF) and their non-transgenic counterparts were used. The animals weighed approximately 20 grams and were about sixteen weeks old. Production of the transgenic mice was accomplished by injecting mouse ova with the entire genomic DNA encoding for hCD55 using yeast artificial chromosomes (YAC), as described by Yannoutsos et al.(14). The hCD55-mouse strain used, contained 10 integrated copies in the genome and the expression of the relevant protein on spleen, kidney, heart, liver and lung was verified by anti-hCD55(15). Splenocytes of mice transgenic for hDAF showed a strong expression of the transgene by FACS analysis. The animals were housed in a certified animal breeding facility under standard conditions and had free access to standard mouse chow (AM II, Hope Farms, Woerden, The Netherlands) and water acidified to pH 2.2 ad libitum. The experimental protocols adhered to the rules laid down in the Dutch Animal Experimentation Act (1977) and the published Guidelines on the Protection of Experimental Animals by the Council of the EC (1986). The Committee on Animal Research of the Erasmus university Medical Centre Rotterdam, The Netherlands had given permission to perform the experimental protocols.

Experimental groups

Group	NaCl or CoPP	KH perfusion	Ischemia	KH reperfusion	HS perfusion	N
1. controls	NaCl	Yes	Yes	Yes	No	13
2. controls	CoPP	Yes	Yes	Yes	No	12
3. controls	NaCl	Yes	No	No	Yes	6
4. hCD55	NaCl	Yes	No	No	Yes	4
5. controls	CoPP	Yes	No	No	Yes	6
6. hCD55	CoPP	Yes	No	No	Yes	5

KH = Krebs-Henseleit perfusion; HS = human serum perfusion.

Mice were pre-treated with NaCl (no upregulation of HO-1) or CoPP (upregulation of HO-1). Groups 1 and 2: 10 minutes perfusion with KH, 30 minutes ischemia and 45 minutes reperfusion with KH. Groups 3,4,5 and 6: 10 minutes perfusion with KH, no ischemia, perfusion with human serum until cessation of heart beats occurred.

The Langendorff circuit

16 hours before the experiments, mice were injected with 0.6 mL of a single dose of either Cobalt protoporphyrin (CoPP) (3 mg/kg) or NaCl (0.9%) intraperitoneally. All mice were anaesthetised with isoflurane inhalation prior to the surgical procedure. The abdominal wall was opened and 0.6 mL Heparin (50 IU/mL) was injected into the abdominal inferior vena cava. Via two lateral incisions the thorax was opened and the heart was removed quickly. A 22-gauge indwelling cannula was inserted into the ascending aorta and secured with a 4.0 ligature. The heart was directly perfused with oxygenated Krebs-Henseleit-Haemaccel solution using a syringe and linked to the Langendorff device, as described previously (16). Acclimatisation of in all groups was accomplished by perfusion with Krebs-Henseleit-Haemaccel solution for 10 minutes. In the first two experimental groups, 30 minutes of warm ischemia was given by interruption of KH perfusion. Thereafter, 45 minutes of reperfusion was given (17). Failure of the heart was manifest when the heart was unable to regain its contractions during reperfusion. Irrespective of heart performance, the experiment was not ended until 45 minutes of reperfusion. After the initiate 10 minutes of perfusion with KH in groups 3,4,5 and 6, no ischemic period was included, but we directly switched to 15% human serum perfusion. Survival time was defined as the time between the start of serum perfusion and complete cessation of heartbeats. This was monitored by using multichannel registration. Heart rate and flow were registered as well.

Human serum

Frozen, pooled, human O-type plasma was obtained from the blood bank of the University Hospital Dijkzigt (Rotterdam, The Netherlands). Human serum was made out of human plasma by adding 0.5 mL Thrombin to 200 mL human plasma, inducing clotting. After removal of the clot, the serum was diluted with Krebs-Henseleit-Haemaccel solution to obtain the 15% perfusion concentration. For each experiment fresh human serum was prepared.

Intraperitoneal Cobalt protoporphyrin IX (CoPP) and NaCl injection

CoPP was dissolved in 0.2 M NaOH and adjusted to pH 7.4. The final volume was made up with 0.9% NaCl. Before each experiment fresh CoPP and NaCl solutions were prepared.

Sixteen hours before each experiment, control mice received 0.6 mL of 0.9% NaCl, and experimental mice were injected with 0.6 mL CoPP (3 mg/kg), intraperitoneally. CoPP at this dose has been shown to upregulate HO-1 in vivo during 24 hours (7;12).

Determination of Heme Oxygenase-1 (HO-1) expression

Immunohistochemistry

HO-1 expression was evaluated by immunohistochemical staining of the heart tissues with polyclonal goat anti-HO-1 antibody. Hearts were sliced in sections of 7 μm with a cryostat and stored at -20°C . Later these tissue sections were air-dried for 30 minutes, after which they were fixed in acetone for 10 minutes at 4°C . After air-drying for 10 minutes, the endogenous peroxidase activity was blocked with 0.05% H_2O_2 in PBS solution for 15 minutes. The sections were incubated with a 1:10 dilution of the primary antibody (goat-anti-HO-1) for 1 hour. Control sections were incubated with PBS. After washing with Phosphate Buffered Saline / Bovine Serum Albumine (PBS/BSA) (3x2 minutes), the sections were incubated with a 1:200 dilution of the secondary antibody (rabbit anti-goat immunoglobulins) for 30 minutes. Sections were washed with PBS/BSA (3x2 minutes), and incubated with a 1:100 dilution of the tertiary antibody (PAP (goat)) for 30 minutes. The sections were washed with PBS/BSA (3x2 minutes), after which peroxidase activity was visualized with di-amino-benzidin (DAB) Substrate-Chromogen solution. The tissue sections were rinsed of with water, fixed in formaldehyde and counterstained with Mayer's Hematoxylin, and again rinsed of with water. Finally, the sections were dehydrated using a series of 70%, 90% and 100% ethanol, washed in Xylol, and covered with a coverslip.

Northern blot analysis

Total RNA was isolated from hearts of mice, either injected with NaCl (no upregulation of HO-1) or CoPP (upregulation of HO-1) using the RNAsol method. Total RNA was isolated from hearts of mice, either injected with NaCl or CoPP. Heart tissue was freshly collected in a polypropylene tube, 2 mL RNAsol B Homogenate (1mL / 10 mg heart tissue) was added, whereafter the heart tissue was crushed with a sonicator. To collect RNA, 1 ml homogenate was mixed with 100 μl chloroform, and centrifuged at $12000 \times g$ for 15 minutes at 4°C . The supernatant was discarded, 500 μl phenol was added, and centrifuged. The supernatant was removed, 500 μl chloroform was added and again it was centrifuged, after which the supernatant was removed. To precipitate the RNA 500 μl isopropanol was added and this mixture was stored at -20°C for 30 minutes, after which centrifuged at $12000 \times g$ for 30 minutes at 4°C . The supernatant was thrown away and a pellet has appeared, which contains

RNA. 1 ml 75% ethanol was added to the pellet and centrifuged at 7500*g for 8 minutes at 4°C. The supernatant was thrown away and the pellet was air-dried for 10-15 minutes. To dissolve the RNA, 50µl sterile water was added to the pellet and incubated in a water bath (60°C) for 15 minutes. After this period, the sample was put on ice again. To measure the amount of RNA in a photospectrometer (E260, E280, E320), 4 µl of the sample was mixed with 996 µl sterile water. When enough RNA was isolated, 20 µl sample buffer (SB) was added and incubated in a 65°C water bath for 10-15 minutes. Finally, 1 µl ethidium bromide was added. Now the RNA sample was ready for electrophoresis. RNA was fractionated on a formaldehyde/1.0-1.5% agarose gel overnight at 25 V, and transferred to hybridization nylon transfer membrane. After washing the membrane with 2xSSC it was baked at 80°C for 2 hours, prehybridized for 6-12 hours at 42°C with 10 ml of hybridization solution (containing 9% dextran sulphate, 1M NaCl, 10x Denhardt's, 100 µg/ml denatured fragmented salmon sperm DNA, 50% formamide, 50mM TRIS pH 7.5, 0.5% SDS), and hybridized for 12-16 hours at 42°C in 5 ml of the above hybridization solution.

A HO-1 probe of 600 base pairs (bp), representing nucleotides +628 to +1132, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe were labeled with [α 32P]dATP (18-20). The membrane was washed with 2xSSC, air-dried, and exposed to x-ray film (Kodak) with intensifying screen at -80°C. Northern blots were quantified densitometrically by use of a Storm Phosphor Imager. Relative mRNA levels were calculated after correcting for RNA loading by normalizing the hybridization signal intensity with the GAPDH signal. After signal quantization, the membrane was kept for later rehybridizations (21).

Infarct size measurement by 2,3,5-triphenyltetrazolium chloride (TTC) staining

In preparation of the 2,3,5-triphenyltetrazolium chloride solution, the following protocol was used. Stock A: 2.04255 g KH_2PO_4 was dissolved in 150 mL sterile water. Stock B: 8.019 g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ was dissolved in 450 mL sterile water. 100 mM phosphate buffer was made by adding 400 mL of stock B to 100 mL of stock A. The buffer was ready to use when pH was between 7.0 and 7.4. To prepare a 2% TTC-solution, 2 g of TTC was added in 100 mL phosphatebuffer. TTC staining was performed on all hearts. After 45 minutes of reperfusion with KH or after switching to perfusion with human serum, the heart was detached from the Langendorff. A syringe containing 2 ml of TTC solution was attached to the cannula, and the heart was slowly flushed. Each heart was sectioned transversely into four parts. Areas that appeared pale white after TTC staining were ischemic, whereas viable myocardium appears red (22;23). Computer analysis (Research Assistant) was performed on the two middle

transverse sections of these four parts. The infarct size was measured as a percentage of the complete section. A mean percentage of the infarct size was calculated.

Statistical analysis

Data from individual experiments are reported as means \pm standard deviation (SD). The non-parametric Mann-Whitney and Chi-Square test, and Independent Student *t* test were used to analyse the data. Differences were considered significant when $P < .05$.

Results

Induction of Heme oxygenase by CoPP

Immunohistochemical staining of the hearts with anti-HO-1 antibody showed HO-1 deposition on the vascular endothelium and along the cardiomyocytes, in both CoPP pretreated animals and controls (figure 1). In hCD55 transgenic and non-transgenic mice, pretreated with CoPP, Northern blot analysis showed that HO-1 mRNA was significantly increased as compared to transgenic and non-transgenic controls (figure 3). Densitometric quantitation of relative HO-1 mRNA levels in cells exposed to CoPP showed a tenfold increase in HO-1 mRNA in group B as compared to group A. Between group C and D there was an eightfold increase.

Heart perfusion and recovery of contractility

In KH perfused hearts, a significant difference was detected between control mice (group 1), in which 6 out of 13 hearts were unable to regain contractions, and HO-1 induced mice (group 2), in which 3 out of 12 hearts were unable to regain contractions ($P = .018$). Figure 2 shows the difference in flow after 30 minutes of warm ischemia in group 1 and 2. A significantly better recovery of the flow in the HO-1 induced mice (group 2) ($P = .020$) was found, indicating that the difference in flow before and after the ischemic period in the CoPP pretreated hearts was smaller than in group 1. As shown in table 1, we observed a significant difference in heart survival between hCD55 transgenic mice and non-transgenic controls perfused with human serum, in both NaCl and CoPP injected animals ($P = .030$ and $P = .041$, respectively). Within the transgenic and non-transgenic groups, no differences were found between control mice and HO-1 induced mice.

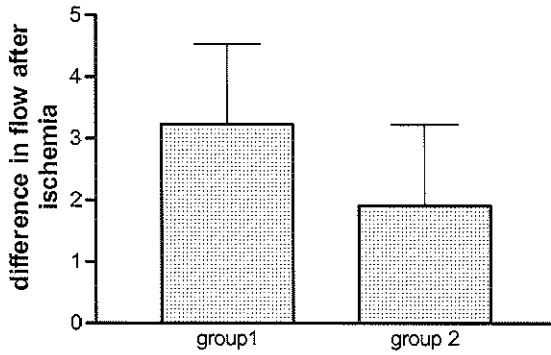


Figure 2. The difference in flow ($\text{flow}_{\text{before}}$ minus $\text{flow}_{\text{after}}$) after 30 minutes of warm ischemia ($P = .020$). Hearts were perfused with Krebs-Henseleit (KH) for 10 minutes, then 30 minutes of warm ischemia was given, and 45 minutes of reperfusion with KH. Group 1: controls, pretreated with NaCl; group 2: controls, pretreated with CoPP.

Table 1. Mean survival times of hCD55 and control mice, pretreated with NaCl or CoPP, perfused with human serum (HS) in the Langendorff system

Group	Mean survival times (minutes)	Average
3. controls + NaCl + HS	3-4-4-6-6-9	5.3
4. hCD55 + NaCl + HS	6-10-15-18	12.3*
5. controls + CoPP + HS	3-5-5-5-6-9	5.5
6. hCD55 + CoPP + HS	6-8-8-9-11	8.4**

* $P = .030$ for group 3 vs 4, and ** $P = .041$ for group 5 vs 6.

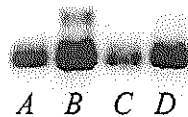


Figure 3. Northern blot of control mice injected with CoPP (D), hCD55 mice injected with CoPP (B), control mice injected with NaCl (C), and hCD55 mice injected with NaCl (A). Densitometric quantitation of relative HO-1 mRNA levels exposed to CoPP showed a tenfold increase in HO-1 mRNA in B compared to A (8.3% versus 0.8%). Between C and D there was an eightfold increase (0.4% versus 3.5%).

Figure 1. Immunohistochemical staining of the hearts with anti-HO-1 antibody showed a similar intensity of HO-1 deposition, on the vascular endothelium and along the cardiomyocytes, in NaCl and CoPP pretreated non-transgenic mice. A: negative control, B: NaCl pretreated mouse heart, C: CoPP pretreated mouse heart (160x).

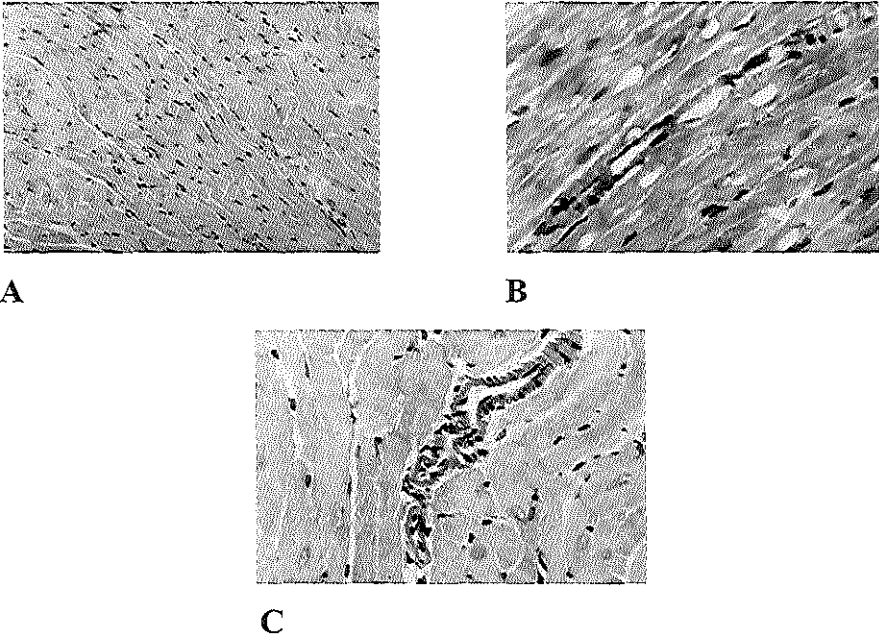
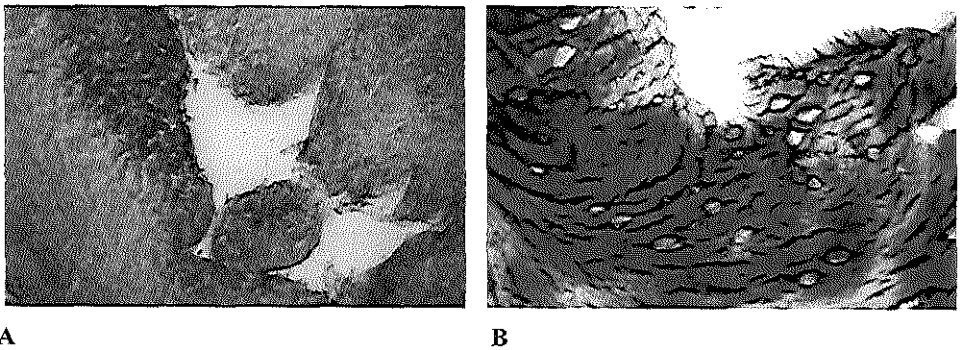


Figure 4. Infarct size after TTC staining in a control (A: NaCl pretreated) and a HO-1 induced mouse heart (B: CoPP pretreated). White areas are infarcted, red areas show vital heart tissue (160x).



Infarct size

A part of the results is shown in figure 4, 5 and 6. After 30 minutes of warm ischemia (figure 5), a highly significant decrease in the infarct size of the heart tissues could be demonstrated in response to upregulation of HO-1 by CoPP (group 2), as compared to controls (group 1) ($P = .000$) (figure 4). Without 30 minutes of warm ischemia (figure 6), the infarct size was also clearly reduced in both CoPP pretreated controls and hCD55 mice (group 5 and 6), as compared to controls and hCD55 mice pretreated with NaCl (group 3 and 4) ($P = .004$ and $P = .014$, respectively). hCD55 transgenic mice injected with NaCl and perfused with human serum (group 4) showed a significant reduction in infarct size as compared to non-transgenic controls (group 3) ($P = .042$). Although the survival times between group 5 and 6 did differ, TTC staining in hCD55 transgenic mice pretreated with CoPP (group 6) was similar as in control hearts (group 5).

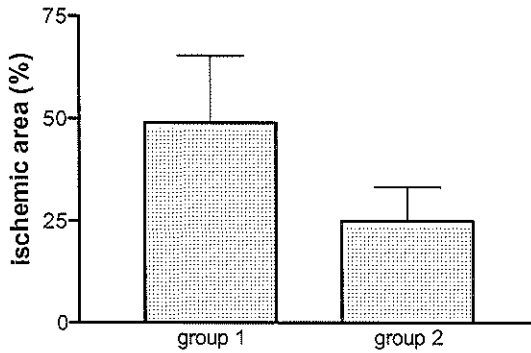


Figure 5. Infarct size measurement in non-transgenic controls after 30 minutes of warm ischemia, either pretreated with NaCl (group 1) or with CoPP (group 2). After perfusion in the Langendorff device, a syringe containing 2 ml of 2% TTC solution was attached to the cannula, and the heart was slowly flushed. Each heart was sectioned transversely into four parts. Areas that appear pale white after TTC staining were ischemic, whereas viable myocardium appears red. Computer analysis was performed on the two middle transverse sections. The infarct size was measured as a percentage of the complete section. A mean percentage of the infarct size was calculated. The mean infarct size in group 1 was 49% versus 25% in group 2 ($P = .000$).

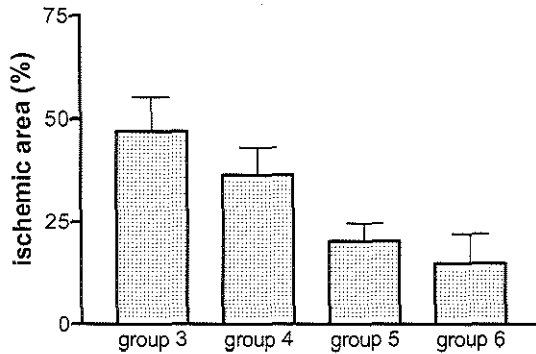


Figure 6. Infarct size measurement in hCD55 mice and non-transgenic controls without 30 minutes of warm ischemia, either pretreated with NaCl (group 3 and 4) or with CoPP (group 5 and 6). After perfusion in the Langendorff device, a syringe containing 2 ml of 2% TTC solution was attached to the cannula, and the heart was slowly flushed. Each heart was sectioned transversely into four parts. Areas that appear pale white after TTC staining were ischemic, whereas viable myocardium appears red. Computer analysis was performed on the two middle transverse sections. The infarct size was measured as a percentage of the complete section. A mean percentage of the infarct size was calculated. The mean infarct size in groups 3,4,5 and 6 was 47%, 36%, 20% and 15%, respectively (group 3 vs 4, $P = .042$; group 3 vs 5, $P = .004$; group 4 vs 6, $P = .014$).

Discussion

HO-1 is a stress enzyme which catalyzes the degradation of heme. All end products of heme degradation, including biliverdin, bilirubin, CO and free iron, are known to modulate immune functions. Biliverdin and bilirubin act as potent free radical scavengers, CO contributes to endothelium-dependent vasodilatation and inhibits platelet aggregation, whereas free iron regulates the expression of ferritin, a cytoprotective gene. All these end products may have a positive influence on the viability of transplanted tissue. Upregulation of HO-1 leads to cytoprotection and reduction of certain immune responses, such as IFN γ - and IL-2-production and may provide a novel strategy to diminish xenograft rejection. We first examined the contribution of HO-1 on the recovery of myocardial function after warm ischemia, and secondly, on the heart survival during xenoperfusion. We and others (14;24-27) have shown that complement regulation at the level of C3 leads to an increased graft survival when hearts are ex vivo perfused with human serum.

Northern blot analysis confirmed the upregulation of HO-1 mRNA in mouse hearts after pretreatment with CoPP. In the present experiments, we showed that upregulation of HO-1 protects the heart subjected to ischemia/reperfusion injury, as marked by a reduced infarct size, and a better recovery of myocardial function after 30 minutes of warm ischemia. This finding corresponds to previous observations by others (8;9). We found that after a warm ischemic period of 30 minutes, 6 out of 13 hearts failed to start beating again in the control group. In the HO-1 group only 3 out of 12 hearts failed, which indicates that upregulation of HO-1 improves the performance of the heart. However, further study using hCD55 transgenic hearts (group 6) revealed that upregulation of HO-1 did not result in an additional effect on the prolongation of xenograft survival. In a study by Clarck et al. (17), an increased recovery of postischemic myocardial function in the rat was found after HO-1 induction with hemin 24 hours before ischemia. In another study performed by Soares et al. (13), it was also found that expression of HO-1 in a mouse-to-rat cardiac xenograft model is associated with xenograft survival (26). Furthermore, both Clarck et al. and Soares et al. evaluated heart performance in view of more specific parameters. We draw conclusions based on infarct size, myocardial function following 30 minutes of warm ischemia, heart rate, flow rate and heart beats. Our findings in the Langendorff model do not support the possibility for HO-1 to improve the overall xenograft survival. But in the warm ischemic groups upregulation of HO-1 has a positive effect on regaining heart contractions, flow recovery and reduction of the infarct size. In the groups without warm ischemia, HO-1 also significantly reduces the infarct size, but does not prolong graft survival. Therefore, we postulate that in the Langendorff model upregulation of HO-1 may contribute to the vitality of the transplanted organ, but has no additional effect on graft survival. Moreover, that there is no correlation between the infarct size and the prolongation of xenograft survival.

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CHAPTER 10

SUMMARY & CONCLUSIONS

1.1 Summary and conclusions

This thesis describes investigations related to the efficacy of human complement regulatory proteins in hyperacute rejection of cardiac xenografts. In *Chapter 1* an overview is given on several aspects of transplantation in general, and xenotransplantation in particular. Due to the difference in demand and supply of donor organs and the mortality of patients awaiting a donor organ, medical science is trying to find an answer to overcome the problem of organ shortage. Xenotransplantation, i.e. transplantation of cells, tissues or organs over a species barrier, may be a possible solution for the donor shortage. Xenotransplantation can be seen either as a permanent replacement of the diseased organ or as a temporary therapy awaiting a suitable human donor. However, as xenotransplantation may not become a clinical reality due to ethical reasons and virological problems, there are several alternatives. Bioartificial organs can be used to support the diseased organ. Stem cell transplantation, cloning of cells / tissues / organs, and gene therapy may be used to produce (parts of) organs. These new techniques are still in the early phases, but develop furiously.

The emphasis in this thesis reclines on the protection of cardiac xenografts against HAR by genetic modification of the organ donor. Three human complement regulatory proteins are known to inhibit the activation of the complement cascade. We have investigated the effects of these hCRPs on protecting grafts against HAR not only in the isolated heart perfusion model, i.e. the Langendorff device, but in a rodent-to-primate transplantation model as well. Moreover, since ischemia reperfusion injury may contribute to graft dysfunction and loss, we examined the additional effect of upregulation of HO-1, which diminishes ischemia reperfusion injury, on the graft survival of hCD55 transgenic mouse hearts in the Langendorff circuit. Furthermore, we studied the early cellular rejection process (i.e. PMN infiltration) by perfusing hCD55 rat hearts with human blood in the Langendorff system. Finally, we studied whether NK cells and macrophages contributed to the late rejection process in the guinea pig-to-rat model.

We demonstrated that particularly regulation at the level of C3 leads to the desired effect: mouse hearts transgenic for hCD46 (MCP) showed a marked increased survival time when perfused with human serum, whereas hCD59 transgenes did not. In *Chapter 3* we investigated whether expression of a second CRP (hCD55) would further improve xenograft survival. Therefore, hearts were first perfused with Krebs-Henseleit and after 10 minutes switched to perfusion with 15% human serum using a Langendorff device. Rejection was defined as complete cessation of heartbeats. The results indicated that hCD46 hearts show a 3-fold

increase in survival over controls. Complement regulation at the C3 level by hCD55 led to similar prolongation as regulation by hCD46. Expression of both hCD46 and hCD55 did not further enhance survival time. It thus appears that, as far as protection against HAR is concerned, inhibition at C3 by one CRP provides the maximal attainable effect.

In *Chapter 4* the survival of single and double transgenic mouse hearts in the Langendorff circuit by means of ex vivo heart perfusion with human serum was compared. Again we found that hCD46 or hCD55 mouse hearts are protected against HAR, whereas hCD59 hearts are not. Furthermore, hCD59xhCD55 and hCD59xhCD46 double transgenic mouse hearts are better protected against HAR than their controls, but not better than single transgenic mouse hearts (hCD55 or hCD46). Surprisingly, the combination hCD55xhCD46 was even worse than hCD55 or hCD46 alone. The main conclusion of this study was that to prevent HAR it is enough to be transgenic for one complement regulatory protein, which inhibits at the level of C3.

In *Chapter 5* we compared hDAF transgenic mouse hearts perfused with human serum in the Langendorff device and hDAF mouse hearts transplanted into cynomolgus monkeys. The first aim of this study was to determine whether perfusion of mouse hearts with higher human serum concentrations or human blood might explain some of the differences found in survival time of the performed experiments with rat heart xenografts (*Chapter 6*). Secondly, we investigated whether the observed differences in survival times of rat xenografts between in vivo and ex vivo transplantation would also hold for mouse hearts transgenic for hDAF. In conclusion, in the ex vivo situation, the efficacy of hDAF transgenesis in preventing HAR is limited by serum complement concentration. In the ex vivo heart perfusion model both homozygous and heterozygous hDAF mouse hearts survived longer as non-transgenic controls. However, in both in vivo and ex vivo model the amount of polymorphonuclear (PMN) adhering to the vascular endothelium was significantly lower in hDAF transgenes as compared to their controls.

In *Chapter 6* we evaluated the resistance of hDAF transgenic rat hearts against complement-mediated damage by perfusion with human serum in the Langendorff device and by transplanting hDAF rat hearts into cynomolgus monkeys. Hemizygous transgenic hearts perfused with human serum showed normal function for more than 60 minutes. Hemi- and homozygous transgenic hearts transplanted into primates showed longer survival than controls. In addition, homozygous hearts survived longer than hemizygous, however,

homozygous rejected hearts expressed signs of AVR. In conclusion, endothelial-specific DAF expression increased the heart transplant survival in a rat-to-primate model.

In the Langendorff model we have demonstrated that rat hearts transgenic for human CD55 (hCD55), perfused with human serum were protected against HAR. However, in the ex vivo situation these hearts were found to be destroyed by a process occurring after the period of HAR. In *Chapter 7*, we questioned whether transgenic hearts for hCD55 are also protected against adhesion and infiltration by cells implicated in the early phases of xenograft rejection. Therefore, transgenic rat hearts and their controls were perfused with either heat-inactivated or normal human blood solutions for 60 minutes. The results indicated that independent of the presence of C, hCD55 transgenic hearts revealed less adhering PMN-leukocytes to the endothelium as compared to controls (31% versus 60%). Furthermore, hCD55 expressed on rat hearts does not only inhibit complement activation, but also human leukocyte-adhesion. Apparently it functions as an anti-adhesion molecule. hCD55 is not only an efficient factor in protecting grafts against HAR, but protects the graft against adhesion of leukocytes as well.

Hyperacute rejection (HAR) of a discordant xenograft can be avoided by complement manipulation, but acute vascular rejection (AVR) still leads to graft loss. It is generally assumed that macrophages and NK cells play key roles in AVR. In the study described in *Chapter 8* the survival times and cellular infiltrate following guinea pig to rat heart transplantation was analyzed in the course of AVR, following aspecific and specific manipulation of macrophages and NK cells. HAR was overcome by a single injection of cobra venom factor 1 day before heart transplantation. To aspecifically reduce the inflammatory response dominating AVR, dexamethasone (DEXA) was given. Treatment with DEXA markedly reduced infiltration by NK cells, macrophages, and granulocytes. It also led to prolonged graft survival times. In the second series of experiments the specific roles of NK cells and macrophages in AVR were further assessed. Monoclonal antibody 3.2.3 was used to selectively deplete NK cells. Liposome-encapsulated dichloromethylene-biphosphonate was given to achieve macrophage depletion. Neither of these specific treatments, alone or combined, led to prolonged graft survival. Immunohistology revealed that at day 2 after transplantation no NK cells or macrophages were present in grafts from the combined treatment group. Only a mild infiltration of granulocytes was observed. Collectively, these results strongly suggest that NK cells and macrophages are not likely to be pivotal cell types in AVR.

Ischemia reperfusion injury may play a pivotal role in graft dysfunction and organ loss. In *Chapter 9*, we examined the effect of upregulation of HO-1, a molecule known to diminish I/R injury on the improvement of the performance of mouse hearts perfused with human serum in the Langendorff circuit. Our findings indicate that upregulation of HO-1 protects mouse hearts from ischemic injury by reducing the infarct size. Furthermore, HO-1 improves the performance of the hearts, however, an additional effect of HO-1 on the prolongation of xenograft survival could not be detected.

Concluding remarks

This dissertation discusses the role and efficacy of human complement regulatory proteins in hyperacute rejection of cardiac xenografts. In discordant xenogeneic transplantation models, HAR is the main cause of graft failure. HAR is mediated by the binding of naturally occurring antibodies and complement activation. Complement activation is normally regulated by regulatory factors, i.e. CD55, CD46, both regulators at C3 level, and CD59, regulator at C9 level. To prevent HAR, it appears that manipulation at the level of complement factor C3 with one CRP, i.e. CD55 or CD46, provides the maximal attainable effect. Combinations of 2 CRPs, either inhibiting at C3 level, or both at C3 and C9 level, does not add to the beneficial effect of only one CRP, mediating C3. Moreover, we have found that hCD59 does not protect mouse hearts against HAR, but this may be due to the lower expression level of CD59 as compared to CD55 and CD46. Thus, the amount of expression of CRP is important. Organs which overexpress CRP are better protected against HAR than organs with low levels of CRP. This phenomenon has been illustrated by the following: hearts from mice expressing similar levels of pig CD59 or human CD59 were perfused *ex vivo* with human serum. When highly expressed on endothelium in transgenic mice, pig CD59 provided equivalent protection to human CD59 in a model of human complement-mediated xenograft rejection. Thus supranormal expression of endogenous porcine CRPs may be a feasible alternative to the expression of human CRPs in preventing HAR of pig-to-primate xenografts (1).

It is dubious that xenogeneic heart transplantation will become clinical practice without further genetic modification to overcome the immunological barriers. We have investigated whether other contributing factors, like Heme oxygenase-1 (HO-1), would improve xenograft survival, but in our Langendorff model HO-1 did not add to the advantageous effect of hCD55 transgenic mouse hearts. In pigs, Gal α 1-3Gal knockout techniques are not available yet (2), but they will be in the near future. If the knockout techniques could be combined with CRP transgenesis, xenograft survival should improve. However, only 85% of the Gal α 1-3Gal proteins are known, thus the unknown 15% can cause new rejection problems. As progress

will be made in knockout and cloning techniques, this will contribute to a cautious introduction of xenotransplantation in the clinical situation. In the mean time, we must focus on the physiological and biochemical imbalances in heart function between pig and man, the virological problems and last but not least, the ethical considerations.

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CHAPTER 11

SAMENVATTING &

CONCLUSIES

1.1 Samenvatting en conclusies

Deze dissertatie beschrijft het onderzoek gerelateerd aan het nut van humane complement regulerende eiwitten tijdens hyperacute afstoting van cardiale xenotransplantaten. In *Hoofdstuk 1* wordt een overzicht gegeven van de verschillende aspecten van transplantatie in het algemeen, en van xenotransplantatie in het bijzonder. Door de discrepantie in vraag en aanbod van donor organen, en de mortaliteit van patiënten die op een donor orgaan wachten, probeert de gezondheidszorg een antwoord te vinden om het orgaan-tekort op te lossen. Xenotransplantatie, dat wil zeggen de transplantatie van cellen, weefsels of organen over een species barrière, kan een mogelijke oplossing zijn voor het donor tekort. Xenotransplantatie kan gezien worden als een permanente vervanging van het zieke orgaan, of als een tijdelijke therapie tijdens het wachten op een geschikte humane donor. Echter, de mogelijkheid bestaat dat xenotransplantatie, om ethische en virologische redenen, klinisch niet haalbaar is. Dan zijn er verschillende alternatieven. Bioartificiële organen kunnen gebruikt worden om het zieke orgaan te ondersteunen. Stamcel transplantatie, het klonen van cellen / weefsels / organen, en gentherapie kunnen ingezet worden om organen, of delen ervan, te produceren. Deze nieuwe technieken bevinden zich in een vroege ontwikkelingsfase, maar deze ontwikkelingen gaan razend snel.

De nadruk in deze dissertatie ligt op de bescherming van cardiale xenotransplantaten tegen hyperacute afstoting via genetische aanpassing van de orgaan donor. Drie humane complement regulerende eiwitten (hCREs) zijn bekend die de activatie van de complementcascade remmen. We hebben niet alleen de effecten van deze hCREs op de bescherming van het transplantaat tegen hyperacute afstoting in het geïsoleerde hart perfusie model, het zogenaamde Langendorff systeem, onderzocht, maar ook in een knaagdier-naar-primaat transplantatie model. Omdat ischemie reperfusie schade een bijdrage kan leveren aan orgaan transplantaat falen en verlies, hebben we bovendien gekeken naar het additionele effect van opregulatie van HO-1, dat ischemie reperfusie schade vermindert, op de transplantaat overleving van hCD55 transgene muizenharten in het Langendorff model. Verder hebben we de vroege cellulaire afstotingsreactie (PMN infiltratie) onderzocht door hCD55 rattenharten te perfunderen met humaan bloed in de Langendorff opstelling. Uiteindelijk hebben we in het cavia-naar-rat model bestudeerd of NK cellen en macrofagen bijdragen aan het late cellulaire afstotingsproces van een xenotransplantaat.

In de beschreven studies hebben we aangetoond dat hoofdzakelijk regulatie op C3 niveau leidt tot het gewenste effect: namelijk dat muizenharten die transgeen zijn voor hCD46

(MCP) een toename in overlevingstijd lieten zien, als ze geperfundeed werden met humaan serum, terwijl hCD59 transgenen dit niet deden. In *Hoofdstuk 3* onderzochten we of expressie van een tweede CRE, namelijk hCD55, tot een verdere toename van de xenotransplantaat overleving zou leiden. Daarom werden harten eerst geperfundeed met Krebs-Henseleit en na 10 minuten schakelden we om naar perfusie met 15% humaan serum, gebruikmakend van de Langendorff opstelling. In dit model wordt afstoting gedefinieerd als complete uitdoving van hartslagen ('stoppen met kloppen'). De resultaten hielden in dat hCD46 harten een drievoudige toename van de overleving lieten zien in vergelijking met controles. Complement regulatie op C3 niveau door hCD55 leidde tot vergelijkbare verlenging als regulatie door hCD46. Expressie van beide eiwitten, hCD46 en hCD55, leidde niet tot verlenging van overleving. Het bleek dus, voor zover het bescherming tegen hyperacute afstoting betrof, dat remming op C3 niveau door één CRE voor het maximaal, haalbare effect zorgde.

In *Hoofdstuk 4* is de overleving van enkel en dubbel transgene muizenharten in het Langendorff model vergeleken door ex vivo hart perfusie met humaan serum. Weer vonden we dat hCD46 of hCD55 muizenharten beschermd zijn tegen hyperacute afstoting, terwijl hCD59 harten dat niet waren. Daarnaast zijn hCD59xhCD55 en hCD59xhCD46 dubbel transgene muizenharten beter beschermd tegen hyperacute afstoting dan hun controles, maar niet beter dan enkel transgene muizenharten (hCD55 of hCD46). Verbazingwekkend was het dat harten met de combinatie hCD55xhCD46 zelfs een slechtere overleving hadden dan harten die alleen hCD55 of hCD46 transgeen waren. De belangrijkste conclusie van deze studie was dat het voldoende is om transgeen te zijn voor één complement regulerend eiwit, dat het complement remt op C3 niveau, om hyperacute afstoting te voorkomen.

In *Hoofdstuk 5* hebben we hDAF transgene muizenharten geperfundeed met humaan serum in de Langendorff opstelling en deze vergeleken met hDAF muizenharten die getransplanteerd werden naar cynomolgus apen. Het doel van deze studie was ten eerste het perfunderen van muizenharten met een hogere humaan serum concentratie dan normaal (30% i.p.v. 15%) en met humaan bloed om zodoende misschien de verschillen in overlevingstijd, die we gevonden hadden bij eerdere experimenten met rattenhart xenotransplantaten (*Hoofdstuk 6*), te kunnen verklaren. Ten tweede onderzochten we of de geobserveerde verschillen in overlevingstijd van rattenhart xenotransplantaten tussen in vivo en ex vivo transplantatie ook voor hDAF transgene muizenharten zou gelden. We concludeerden dat in de ex vivo situatie de mate van effectiviteit van hDAF-transgenese ter preventie van hyperacute afstoting gelimiteerd was door de serum complement concentratie. In het ex vivo

hart perfusie model overleefden zowel homozygote en heterozygote hDAF muizenharten langer als niet-transgene controles. Echter, in beide modellen (in vivo en ex vivo) was de hoeveelheid polymorfonucleaire cellen (PMN), die adhereerden aan het vasculaire endotheel, significant lager in hDAF transgene harten in vergelijking met hun controles.

In *Hoofdstuk 6* hebben we de weerstand van hDAF transgene rattenharten tegen complement-gemedieerde schade geëvalueerd door perfusie met humaan serum in het Langendorff model, en door hDAF rattenharten te transplanteren naar cynomolgus apen. Hemizygote transgene harten geperfundeerd met humaan serum lieten een normale functie zien gedurende meer dan 60 minuten. Hemi- of homozygote transgene harten getransplanteerd naar primaten lieten een langere overleving dan controles zien. Weliswaar overleefden homozygote harten langer dan hemizygote, echter, homozygote afgestoten harten lieten verschijnselen van acute vasculaire afstoting zien. We concludeerden dat endotheel-specifieke DAF expressie de hart transplantaat overleving in een rat-naar-primaat model deed toenemen.

In het Langendorff model hebben we gedemonstreerd dat rattenharten transgeen voor humaan CD55 (hCD55), geperfundeerd met humaan serum beschermd zijn tegen hyperacute afstoting. Echter, in de ex vivo situatie werden deze harten vernietigd door een proces dat na de periode van hyperacute afstoting plaatsvond. In *Hoofdstuk 7*, vroegen we ons dan ook af of hCD55 transgene harten ook beschermd zouden zijn tegen adhesie en infiltratie van cellen die betrokken zijn in de vroege fases van xenotransplantaat afstoting. Daarom werden transgene rattenharten en hun controles geperfundeerd met of complement-geïnactiveerd humaan bloed of normaal humaan bloed oplossingen gedurende 60 minuten. De resultaten hielden in dat onafhankelijk van de aanwezigheid van complement, hCD55 transgene harten minder adherentie van PMN-leukocyten aan het endotheel lieten zien in vergelijking met controles (31% versus 60%). hCD55 functioneerde blijkbaar als een anti-adhesie molekuul. In conclusie, hCD55 was niet alleen een efficiënte factor bij de bescherming van transplantaten tegen hyperacute afstoting, maar het beschermde het transplantaat ook tegen adhesie van leukocyten.

Hyperacute afstoting van een discordant xenotransplantaat kan voorkomen worden door complement manipulatie, maar acute vasculaire afstoting leidt nog steeds tot transplantaat verlies. Het wordt algemeen verondersteld dat macrofagen en NK cellen sleutelrollen spelen in acute vasculaire afstoting. In het onderzoek beschreven in *Hoofdstuk 8* zijn de overlevingstijden en cellulaire infiltraten na cavia-naar-rat hart transplantatie geanalyseerd in

de lijn van acute vasculaire afstoting, na aspecifieke en specifieke manipulatie van macrofagen en NK cellen. Hyperacute afstoting werd voorkomen door een enkele injectie met cobragif 1 dag voordat de harttransplantatie plaatsvond. Om de aspecifieke ontstekingsresponse, die centraal staat in acute vasculaire afstoting, te reduceren, werd dexamethason (DEXA) gegeven. Behandeling met DEXA zorgde voor een waarneembare reductie van de infiltratie door NK cellen, macrofagen en granulocyten. Het leidde ook tot verlengde transplantaatoverleving. In de tweede helft van de experimenten werden de specifieke rollen van NK cellen en macrofagen bij acute vasculaire afstoting verder uitgediept. Monoclaal antilichaam 3.2.3 werd gebruikt om selectieve depletie van NK cellen te bewerkstelligen. Liposoom-ingekapselde dichloromechyleen-bifosfonaat werd gegeven om de macrofagen te depletieren. Geen van beide specifieke therapieën, alleen of gecombineerd, leidde tot verlenging van transplantaat-overleving. Immunohistologie liet zien dat na 2 dagen na transplantatie geen NK cellen of macrofagen aanwezig waren in de transplantaten van de gecombineerd behandelde groep. Alleen een milde infiltratie met granulocyten werd geobserveerd. Alles samen genomen suggereerden deze resultaten dat NK cellen en macrofagen niet echt de belangrijkste celtypen zijn bij acute vasculaire afstoting.

Ischemie reperfusie (I/R) schade kan een belangrijke rol spelen in transplantaat dysfunctie en orgaan verlies. In *Hoofdstuk 9*, hebben we het effect van opregulatie van HO-1, een molecuul dat bekend staat om I/R schade te verminderen, bestudeerd op de verbetering van de prestatie van muizenharten geperfundeerd met humaan serum in het Langendorff model. Onze resultaten hielden in dat opregulatie van HO-1 muizenharten beschermde tegen ischemische schade; dit was te zien aan een afname van de infarct grootte. Bovendien verbeterde HO-1 de prestatie van de harten, echter, een additioneel effect van HO-1 op de verlenging van de transplantaatoverleving kon niet worden gedetecteerd.

Curriculum Vitae

Caroline Ada Elisabeth was born on May 29, 1975 in Rotterdam, The Netherlands. In June 1994 she graduated from the Emmaus College (Atheneum / VWO-B) in Rotterdam and in September 1994 she started her medical training at the Erasmus University Rotterdam, where she graduated in July 1998. During her medical study her interest in surgery was stimulated when she worked as a student assistant in the 'studentteam' at the department of Plastic & Reconstructive Surgery (prof. dr. S.E.R. Hovius) of the Academic Hospital Rotterdam Dijkzigt. As part of her fourth year she spent three months investigating a patient with an exceptional congenital malformation of the upper extremity, at the department of Plastic & Reconstructive Surgery as well (mrs. dr. Chr. Vermeij-Keers). During the last three months as a medical student she was offered a PhD-position at the Laboratory for Experimental Surgery (prof.dr. J. Jeekel) of the Erasmus University Rotterdam under supervision of dr. R.L. Marquet and dr. J.N.M. IJzermans. From May 1998 she started working as a PhD-student on the project, entitled 'human complement regulatory proteins in hyperacute rejection of cardiac xenografts'. The project was supported by the Netherlands Heart Foundation (NHF-96.083). In the autumn of 2001 she will start her training period. Her current goal is to obtain her medical degree in the spring of 2003.

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List of abbreviations

AVR	acute vascular rejection
BSA	bovine serum albumine
C	complement
CD	cluster of differentiation
CO	carbon monoxide
CRP	complement regulatory protein
CVF	cobra venom factor
DAF	decay accelerating factor
DEXA	dexamethasone
DNA	deoxyribonucleic acid
DXR	delayed xenograft rejection
EC	endothelial cell
FACS	fluorescence-activated cell sorting
FITC	fluorescein-Iso-Thio-Cyanate
HAR	hyperacute rejection
H&E	hematoxylin eosin
HLA	human leukocyte antigen
HO-1	heme oxygenase-1
HS	human serum
HUVEC	human umbilical vein endothelial cell
ICAM	intercellular adhesion molecule
IFN	interferon
Ig	immunoglobulin
IL	interleukin
I/R	ischemia reperfusion
KH	Krebs-Henseleit
MAb	monoclonal antibody
MAC	membrane attack complex
MCP	membrane cofactor protein
MHC	major histocompatibility complex
MST	mean survival time
NK	natural killer cell
PAF	platelet activating factor
PBS	phosphate-buffered saline
PMN	polymorphonuclear neutrophils
PoERV	porcine endogenous retrovirus
SD	standard deviation
TNF	tumor necrosis factor
TTC	tetrazoliumchloride
vWf	von Willebrand factor
WBC	white blood cell
WT	wild type
XNA	xenogeneic natural antibodies
YAC	yeast artificial chromosomes

List of publications

PROTECTION AGAINST HYPERACUTE XENOGRAFT REJECTION OF TRANSGENIC RAT HEARTS EXPRESSING HUMAN DECAY ACCELERATING FACTOR (DAF) TRANSPLANTED INTO PRIMATES

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NO FUNCTIONAL BENEFIT FOR hDAF-TRANSGENIC RAT LIVERS DESPITE PROTECTION FROM TISSUE DAMAGE FOLLOWING PERFUSION WITH HUMAN SERUM

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