EFFECTS OF ENDOTHELIAL CELL PROTECTION IN INDUCTION OF TRANSPLANTATION TOLERANCE

PhD Thesis

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In search of the “Holy Grail”

Every year, thousands of patients in end stage organ failure receive a new chance at life. These recipients also get a life sentence when they start using immunosuppressive drugs. Researchers and clinicians are working towards achieving success in clinical transplantation tolerance; hence, every small finding is a giant step contributing to pave the way to transplantation tolerance, commonly known as the “Holy Grail” of transplantation medicine.
Abstract

The ultimate goal of clinical transplantation is to achieve an enduring donor-specific allograft acceptance in the absence of long-term immunosuppression. Traditionally, rejection and tolerance of transplanted grafts have been considered within the realm of the adaptive immune system. During the past decade, an increasing body of literature proposes that the innate immune system plays an important role in allograft rejection and transplantation tolerance.

The “danger signals” associated with ischemia/reperfusion (I/R) injury in organ transplantation trigger an early innate immune response. Thus, induction of tolerance is hampered in the clinical setting by circumstances such as prolonged graft ischemia time, which exacerbates I/R injury, characterized by the activation of the graft endothelial cells (EC). Shedding of the graft EC protective layer, particularly heparan sulfate proteoglycan, is central to EC activation, which influences the tolerance-induction mechanisms by provoking strong inflammation. The working hypothesis of the thesis was that reestablishment of the anti-inflammatory and anticoagulatory endothelium using a cytoprotectant may attenuate the early immune response. This may support the strategies aimed at inducing transplantation tolerance.

Treatment of graft endothelium subjected to prolonged ischemia with the EC-protectant low molecular weight dextran sulfate (DXS, MW 5000) restores its quiescent and ‘non-dangerous’ state. Dextran sulfate may reestablish anti-inflammatory and anticoagulatory graft endothelium, possibly by functional replacement of surface protective endothelial glycocalyx. Dextran sulfate treatment significantly reduced EC activation and deposition of complement, as well as infiltration of granulocytes and monocytes/macrophages. The attenuation of innate immune responses by DXS facilitates transplantation tolerance induced by non-depleting anti-CD4 monoclonal antibody in heterotopic cardiac allotransplantation in rats. Moreover, local application of DXS into ischemic vasculature in a rat aortic clamping model modulated mitogen-activated protein kinase (MAPK) activation, reduced complement deposition and preserved the endothelial integrity.

In conclusion, our results demonstrate the role of DXS in ameliorating vascular I/R injury by attenuating the early immune responses. Dextran sulfate may be envisaged as a protectant in I/R injury, which occurs during organ transplantation and other vascular surgical procedures. Furthermore, the attempts to minimize I/R injury appear to be an important approach leading to the successful induction of transplantation tolerance.
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1 Introduction

1.1 Transplantation

Organ transplantation is a promising life-saving therapy for the end-stage failure of organs like kidney, liver, heart and lung. Today, it has been established as a routine medical practice with a significant influence on the survival of patients experiencing end-stage organ failure. The first successful human organ (renal) transplantation was performed by John Merrill, Joseph Murray and Hartwell Harrison at Brigham and Women’s Hospital, Boston, Massachusetts, USA in 1954 (1). Since then, more than half a million organs have been transplanted (http://www.OPTN.org). With the advancement of surgical techniques, immunosuppressive drugs and postoperative care, today organ transplantation has become the golden standard to treat patients experiencing end-stage organ failure. However, while significant progress has been made during the last five decades to prevent acute allograft rejection, most transplanted organs suffer from chronic rejection by the recipient's immune system and only few transplants survive longer than 15 years (2, 3).

1.2 Allorecognition and immune response

1.2.1 Allorecognition

If organ transplantation is performed between genetically identical individuals (syngeneic) or from one individual to itself (autologous), no noticeable immune response occurs due to histocompatibility. In contrast, if transplantation is performed between genetically dissimilar individuals (allogeneic), a significant immune response is elicited due to the foreignness of antigens (alloantigens), which are readily recognized by the recipient immune system. The recognition of donor antigens is termed allorecognition.

The alloantigens are divided into two classes, the Major Histocompatibility Complex (MHC) and Minor Histocompatibility Antigens (mHAg). The principal molecules responsible for allorecognition are coded by the MHC genes, which are the most polymorphic genes in the human genome, making individuals unique. It has been well established that the donor-derived MHC antigens are recognized by the recipient immune system through three distinct pathways. These allorecognition pathways are termed (A) direct (B) indirect and (C) semi-direct pathways (Figure 1). In the direct pathway, alloantigens are recognized via the intact donor-derived MHC molecules, which are expressed on the surface of donor antigen presenting cells (APC).
The intact allogeneic MHC-I and -II molecules are recognized by the recipient CD8⁺ and CD4⁺ T cells, respectively. In the indirect pathway, shed alloantigens are processed and presented to T cells by recipient APC in association with self MHC-II; therefore, the dominant cell types activated in this process are CD4⁺ T cells. The relative contribution of these two pathways in terms of graft rejection is not completely understood. However, the donor antigen recognition through the direct pathway leads to early rejection and the indirect pathway is responsible for the late rejection episodes (4-6). A recently described third pathway is known as the semi-direct pathway (7). In this pathway, recipient dendritic cells (DC) take up intact donor MHC-I and -II molecules from donor DC and endothelial cells and induce antigen-specific recipient T cells.

1.2.2 Alloresponse

Alloresponse is the activation of the recipient immune system after the recognition of donor antigens. The innate and adaptive immune systems orchestrate synergistically to reject the graft through the effector cells and numerous mediators. CD4⁺ T cells are the most important cells in mediating the alloresponse. They secrete cytokines to attract numerous effector cells to orchestrate the immune reactions. These events include contact-dependent CD8⁺ T cell cytotoxicity, macrophage mediated delayed type hypersensitivity, activation of granulocytes and NK cells, antibody production by B cells and the accompanying mediated graft damage.

1.3 Prevention of graft rejection

After the great success of the first renal transplantation in 1954, organ transplantation was established as a promising therapy for end-stage organ failure. However, transplantation was mainly performed between identical twins at that time (8). The major reason for this was the unavailability of remedies to handle the immune responses if performed between allogeneic individuals. Although various approaches to control the immune responses were considered, insufficient knowledge of immunology at that time did not open the window to extend transplantation beyond identical twins. One of the early attempts taken to target suppression of the immune system was reported in 1950 (9). Although this therapy was based on the irradiation of the recipient, the concept encouraged exploration of new therapies to suppress the recipient immune responses. The drugs used to suppress the recipient immune system are known as immunosuppressives. The need to introduce new immunosuppressive drugs increasingly became an important issue in transplantation medicine.
Figure 1. Three pathways of allorecognition. (A) Direct pathway. Donor antigen presenting cells (dAPC) present in the graft migrate to the recipient’s lymph nodes. Recipient CD4+ and CD8+ T cells recognize intact MHC-I and -II on dAPC. The CD8+ T cells require cognate CD4+ help for its full activation, e.g., IL-2. (B) Indirect pathway. Degraded-donor antigens are taken up and processed by recipient antigen presenting cells (rAPC). These antigens are presented to CD4+ T cells in association with self-MHC-II. (C) Semi-direct pathway. The recipient intact MHC-I molecules are internalized and presented to CD8+ T cells and simultaneously, processed donor MHC antigens internalized as necrotic and apoptotic cell materials are presented to CD4+ T cells.

1.3.1 Immunosuppressives

The earliest attempt taken to suppress the rejection process was sub-lethal total body irradiation combined with cortisone therapy (9). Subsequently, a new immune suppression candidate, 6-mercaptopurine, was shown to suppress immune activation in rabbits (10). Later, a variant of 6-mercaptopurine, azathioprine, came into the clinical trials as a promising immunosuppressive drug (11). Simultaneously, the advancement of surgical techniques and the use of different solid organ transplantation came into the clinical arena. Thus, the necessity to suppress the immune response became an important concern in clinical trials. For instance, the first orthotopic heart transplantation (OHT) was established and 102 OHT performed by the end of 1968. However, the mean survival time of those 102 patients was 29 days (12). Azathioprine and prednisone were used as immunosuppressive agents in
those patients. With this therapy, the one-year survival rate increased from 20% to 25% following OHT (13). Prevention of rejection in recipients who received allogeneic grafts was attempted by dual immunosuppression from the 1950s through the 1970s. This two-drug therapy was comprised of a combination of glucocorticoids and azathioprine. These drugs were modestly successful and achieved one-year and five-year renal transplant survival rates of 50% to 60% after living donation and 30% to 50% after deceased donation (14).

The revolutionary breakthrough came with the discovery of cyclosporine in the 1970s in the laboratories of Sandoz in Basel, Switzerland. Introduction of cyclosporine into the clinics in the 1980s dramatically increased the survival rate of transplant patients. Use of cyclosporine as an additional maintenance immunosuppressive agent in combination with azathioprine and glucocorticoids led to a marked reduction in the acute rejection rates. This result boosted the interest in developing newer immunosuppressive medications among pharmaceutical companies. As a result of that, many newer and more potent maintenance immunosuppressive agents were developed, such as FK-506 (tacrolimus) (15). Indeed, immunosuppressives were considered promising candidates in clinical trials. Nevertheless, the dark side of immunosuppressives was also slowly emerging in patients who had been treated with different regimens of immunosuppressive drugs.

1.3.2 Outcome of immunosuppression

Although current immunosuppressive drugs play an important role in clinical transplantation, several major drawbacks are increasingly being considered. Since the drugs have a global immunosuppressive action on the patients, they tremendously enhance the risk of opportunistic infections and malignancies (16, 17). The side effects associated with these drugs are also greater in transplant patients. Some of the notable side effects are nephrotoxicity of calcineurin inhibitors, as well as hypertension and cardiovascular diseases associated with corticosteroids (18-20).

Nevertheless, the current immunosuppressive regimen has had a tremendous impact on the prevention of acute graft rejection, although similar results have not been shown in the prevention of long-term graft rejection. Thus, the graft is at risk of developing chronic graft dysfunction. Transplanted organs have achieved a high early success rate, as exemplified by a 90% one-year survival rate after renal transplantation with current immunosuppressive therapy (www.optn.org/AR2007). Despite this great success in the early phase, long-term survival remains unachievable in clinics. This is a tremendous drawback for transplanted patients, and currently no remedy is available to circumvent this problem. The outcome of chronic graft dysfunction is vasculopathy in the case of the kidney and heart, as the target
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area is the vasculature of these organs. In lungs, the target of chronic rejection is the bronchial tree. Chronic rejection after liver transplantation is rare, as the liver appears to be an immunologically privileged organ and has a high regenerative capacity. Despite the current immunosuppressive therapy, renal and heart allografts are subjected to chronic rejection at a rate of five percent annually (21). Therefore, the necessity to reduce the drawbacks associated with current immunosuppressive therapy is an important factor. At this juncture, induction of transplant tolerance appears as an axiom of faith among clinicians and transplant immunologists.

1.4 Transplantation tolerance

A seminal work by Billingham, Brent and Medawar in Nature entitled “Actively acquired tolerance of foreign cells” has paved the way for the current concept of transplant tolerance (22). Transplantation tolerance is commonly referred to as the “Holy Grail” in transplantation medicine. The accepted definition of transplant tolerance is the absence of destructive immune response to the allograft without need of the immunosuppressive drugs, while maintaining the normal immune response (23).

1.4.1 Mechanism of tolerance induction

Although not mutually exclusive, transplantation tolerance can be divided into central and peripheral tolerance, similar to self-tolerance. Central and peripheral tolerance refers to the thymus and extra-thymic lymphoid tissues, respectively, in terms of the location. Tolerance induction occurs via three basic mechanisms, namely deletion, anergy and suppression/regulation. The relative importance of each mechanism depends on the above-mentioned two locations.

1.4.1.1 Central tolerance

Central tolerance resembles the phenomenon occurring during induction of self-tolerance. Self-reactive T cells in the thymus undergo apoptosis and are removed from the T cell repertoire during fetal development (24). The precursors of T cells migrate from the bone marrow to the thymus, where they proliferate and differentiate. More than 98% of immature T cells undergo apoptosis, depending on the binding affinities of the T cell receptor (TCR) to self-peptides presented by APC (25, 26). Clinically, this type of tolerance can be induced by total body irradiation, which causes irradiation of the mature T cell repertoire. Irradiation is followed by donor bone marrow transplantation, guaranteeing engraftment of donor-derived APC in the
recipient’s thymus, where they are viewed as self. Thus, specific tolerance can be achieved to the donor antigens by the deletion of alloreactive T cells (27). This state is referred to chimerism, in which cells from two genetically different backgrounds co-exist in one individual. Although this helps to induce tolerance, the major clinical limitations are toxicities associated with myeloablative therapy and invasiveness. Moreover, this type of chimerism causes impaired ability to fight infections, due to suppression of T cell immune response (28). To overcome these limitations, mixed-chimerism has been developed. Mixed-allogeneic chimerism can be established by reconstituting the myeloablative individual with both donor- and recipient-derived bone marrow. This individual has both donor and recipient APC (29). However, the clinical applicability of mixed-chimerism is questionable because of the side effects.

1.4.1.2 Peripheral tolerance

Although the frequency is low, mature T cells can escape from the thymic deletion mechanism. These escaped T cells have autoimmune potential; therefore, the mechanisms to induce peripheral tolerance exist to control such T cells. These mechanisms are broadly divided into peripheral deletional and non-deletional. In the deletional category, veto cells and activation-induced cell death (AICD) have been described. It has been demonstrated that veto cells, a type of CD8+ T cell, have the unique function of deleting alloreactive T cells. Veto cells exist in the bone marrow and play a role in bone marrow transplant models. However, the mechanism of alloreactive T cell inactivation/deletion remains elusive (30). Veto cells have been used in some transplantation models, including renal transplantation (30). Activation-induced cell death operates to limit expansion of T cells after being activated upon antigenic stimulation. This can occur by interaction of apoptosis inducing Fas molecule with Fas ligand and also an IL-2 dependent manner.

Anergy is the process in which T cells fail to respond or undergo functional unresponsiveness. This has been demonstrated in vitro by the inability to proliferate and the cytokine production of T cells (31). T cells in the state of anergy are unable to expand clonally in vivo (32). In addition to signaling through TCR, T cells require a second signal, called a costimulatory signal, in order to be fully activated. Costimulatory blockade is considered an important strategy to induce peripheral tolerance in a non-depletion fashion. The binding of costimulatory molecules on APC, like CD80 and CD83, with the CD28 expressed on T cells at the time of antigen presentation, caused the induction of cell proliferation and cytokine production (33). The approaches to block costimulatory molecule engagement lead to an inhibition of alloresponse and T cell proliferation (34). Blockade of CD40 (expressed on APC) and CD40L, also known as CD154 (expressed on T cells), in combination with donor-
specific transfusion caused the inhibition of T cell proliferation in experimental models (35).

There is an emerging consensus that CD4+ T cells play a role in regulatory function in order to establish and maintain tolerance. Infectious tolerance occurs when peripheral immune regulation is mediated by a regulatory type of CD4+ T cells by suppression of effector CD4+ T cells and their conversion into regulatory T cells (Treg) (36). These powerful regulatory CD4+ T cells express CD25 (IL-2R-α) on the surface (37). Hence these cells are known as CD25+CD4+ regulatory T cells, which can be induced using different therapeutic approaches such as anti-CD4 monoclonal antibody (mAb).

1.4.2 Anti-CD4 monoclonal antibody

As a transplantation tolerance induction protocol, CD4 coreceptor targeted mAb therapy has been effectively used in animal models for many years (38, 39). Although the first clinical trial of anti-CD4 mAb was performed in transplantation and autoimmune patients (39, 40), the mechanism of action remains largely unclear. However, extensive research has been done to explore the molecular mechanism of anti-CD4 mAb in different experimental models. Today, anti-CD4 mAb is an effective protocol to induce experimental transplantation tolerance. Recently, humanized non-depleting anti-CD4 mAb have been developed and tested in a phase I clinical trial (41). Anti-CD4 mAb can be divided into depleting and non-depleting antibodies. This grouping is based on the capacity to deplete CD4+ T cells in vivo. Although both depleting (42) and non-depleting antibodies (43) have been used to induce tolerance, depleting antibodies showed a limited therapeutic usage, since they may lead to a prolonged period of immunosuppression and subsequent homeostatic proliferation of T cells.

Non-depleting monoclonal anti-rat CD4 antibody RIB 5/2 was developed for use in different rat transplantation models (44). The applications of RIB 5/2 in different transplantation models led to the exploration of many mechanisms behind transplantation tolerance. RIB 5/2 has been used in corneal allograft (45), heart (46, 47), and kidney (48, 49) transplantation models. It has been proposed that the ligation of the CD4 coreceptor by anti-CD4 mAb induces tolerance by two mechanisms: first by suppressing alloresponse and second by induction of Treg, which may maintain the transplantation tolerance (50).

The mechanism of CD4 mAb has been extensively studied in a TCR transgenic mouse model where all T cells are CD4+ and where no CD8+ T cells or B cells are present. The females of A1.RAG-/- TCR transgenic mice have CD4+ T cells specific
for male Ag (Dby). Female A1.RAG-/- TCR transgenic mice therefore reject male skin grafts in a CD4+ T cell dependent fashion (51). Moreover, these female transgenic mice do not contain detectable levels of CD4+CD25+Foxp3+ regulatory T cells in the thymus or periphery. Treatment with non-depleting anti-CD4 mAb prevented rejection of the skin graft and the female recipients became tolerant for the male skin grafts. Moreover, anti-CD4 mAb therapy caused them to generate and accumulate CD4+CD25+Foxp3+ regulatory T cells in a TGF-β dependent manner (52) (Figure 2).

Figure 2. Induction of tolerance by anti-CD4 monoclonal antibody. (A). T cell activation and immunity. The engagement of antigens in association with MHC-II leads to activation and clonal expansion of T cells. (B). Blockade of CD4 coreceptor. Signal through TCR must be amplified by CD4 coreceptor for the upregulation of costimulatory molecules like CD40L that allow T cell activation. Ligation of CD4 coreceptor leads to blindfolding the immune activation at the initial stage. (C) Maintenance of tolerance. At the later stage, in the presence of transforming growth factor β (TGF-β), which alters TCR signaling threshold, this leads to upregulation of Foxp3. Upregulation of Foxp3 drives T cell differentiation into regulatory T cells, which maintain the tolerance.
1.5 Barriers to transplantation tolerance

Although the tolerance induction protocols have been successfully demonstrated in small animal models, translating these protocols into the clinics may not successful. Pre-existing memory T cells, heterologous immunity and homeostatic proliferation of lymphocytes are considered to be the barriers in the induction of tolerance in the adoptive arm of immune system (53-55). Recently, concern over the innate immune responses in transplantation tolerance has been spurred by an increasing body of literature in which the influence of the innate immune system in tolerance induction has been examined more closely.

The innate immune system has evolved to be the front line of defense to protect the body against invading foreign pathogens. Janeway proposed that the immune system does not respond to all antigens which are foreign to the body, but only to the antigens potentially associated with infections (56), and advanced the “self-non-self discrimination model.”

This idea is consolidated by the discovery of Toll-like receptors (TLR) and other pathogen sensing molecules such as pathogen associated molecular patterns (PAMP) and pattern recognition receptors (PRR) (57, 58). With the discovery of TLR, the role of the innate immune system has been increasingly considered, and the knowledge has been extended to explain molecular mechanisms associated with the sensing of pathogens. Toll-like receptors are a special group of pathogen recognition molecules: this recognition occurs via sensing PAMP (57).

Toll-like receptors are expressed on a wide array of cells in the immune system, mostly APC including dendritic cells, macrophages and B cells. Moreover, they are also expressed on basophils, neutrophils, eosinophils and endothelial cells. Although Janeway’s model explains clearly the discrimination between self and non-self, this model does not consider the signal emanating from any damaged tissues: for example, the ability of the immune system to sense and respond to necrotic cell death. This gap was closed in 1994, when Matzinger proposed the “danger model,” describing the immune surveillance, which detects and responds to microbial infections as well as endogenous alarm signals, collectively known as damage associated molecular pattern (DAMP) (59).
The graft immunogenicity is boosted as a result of I/R injury and BD. The direct influence on toll like receptors (TLR), natural killer cells (NK), and dendritic cells (DC) leads to rapid activation of the innate immunity, which links with the adoptive arm of the immune system and thereby prevents tolerance induction mechanism. Figure is adapted from Kim et al. (60).

Although in evolutionary terms, the innate immune system is older than the adaptive immune system, recognition of the role of innate immunity is new in transplantation. The innate immune system-mediated responses are critical to trigger an early inflammatory reaction following engraftment; thus, the antigen-independent insults have been increasingly focused in research on allograft rejection and tolerance. There is growing evidence that antigen independent factors, such as ischemia/reperfusion (I/R) injury and brain death, critically influence allograft rejection. These antigen independent factors have a remarkable impact on the organ quality and also boost immunogenicity of the graft (Figure 3). The latter has a critical influence on induction of transplantation tolerance through activation of the innate immune responses.

Figure 3. Effect of ischemia/reperfusion (I/R) injury and brain death (BD) on transplantation tolerance. The graft immunogenicity is boosted as a result of I/R injury and BD. The direct influence on toll like receptors (TLR), natural killer cells (NK), and dendritic cells (DC) leads to rapid activation of the innate immunity, which links with the adoptive arm of the immune system and thereby prevents tolerance induction mechanism. Figure is adapted from Kim et al. (60).
1.6 Ischemia/reperfusion injury

Organ injuries occurring after reperfusion of previously viable ischemic tissues are defined as I/R injuries. The consequences of depriving an organ of its blood supply have been recognized as a critical factor in the clinical outcome of organ transplantation. Although the restoration of blood flow to an ischemic organ is essential to prevent irreversible tissue injury, reperfusion itself may amplify tissue injury in excess of that produced by ischemia alone (61). The early clinical evidence between innate alloimmunity and I/R injury came in 1994 with the publication of clinical data from the Munich superoxide dismutase trial. The results of the clinical trial suggest that renal allograft I/R injury initiates acute allograft rejection and contributes to development of chronic rejection (62). Further studies have demonstrated that prolonged ischemia and its manifestation as delayed graft function are associated with reducing graft survival rate (63). Allograft endothelium is the anatomical location most vulnerable to I/R injury. This phenomenon is commonly considered as activation of the graft endothelial cells (EC) as a consequence of I/R injury.

1.6.1 Activation of graft endothelial cells

Vascular EC appear as the foremost interface between donor and recipient and play a key role between the donor organ and recipient immune system. Moreover, they exist as a barrier between the donor organ and the circulating immunocompetent cells of the recipient. Activation of graft EC due to I/R injury plays a central role in initiating an early allograft inflammation. The activation of graft EC has been demonstrated in I/R injury (64-66). Activation of EC is not precisely defined, but tremendous changes occur during I/R injury, which lead to several noticeable changes of graft endothelium. The major changes are loss of cell integrity, expression of leukocyte adhesion molecules, phenotypic changes, cytokine production and upregulation of MHC antigens (Figure 4).

Ischemia/reperfusion injury induces an inflammatory reaction, which promotes chronic rejection and jeopardizes allograft survival (67, 68). It has been shown that significant upregulation of ICAM-1, P- and E-selectin as well as costimulatory molecules, particularly CD80 and CD86, occurs in activated endothelium (69-71). Upregulation of leukocyte adhesion molecules allows leukocytes to adhere and transmigrate to the tissues of graft (72). Recruitment of leukocytes involves their rolling along the endothelium, followed by firm adhesion. The rolling process is mediated in part by EC expressed selectin. Leukocytes utilize integrins such as LFA-1, MAC-1 and VLA-1 for firm adhesion with EC expressed ICAM-1 and VCAM-1. Thus, I/R related EC activation promotes recruitment of leukocytes to the allograft.
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Figure 4. Activation of endothelial cells and role of the glycocalyx. Quiescent endothelium with intact glycocalyx serves to maintain vascular homeostasis. Ischemia/reperfusion injury causes activation of endothelial cells, characterized by perturbation of the glycocalyx and resulting in a proinflammatory and procoagulatory state. The consequences of glycocalyx perturbation are increased vascular permeability, upregulation of intracellular adhesion molecules (ICAM-1), cell adhesion, loss of antioxidative properties by releasing superoxide dismutase (SOD), activation of coagulation by loss of antithrombin III (AT III) and activation of complement pathways. In addition, the glycocalyx helps to regulate endothelial nitric oxide, platelet activation and physical barrier function for macromolecules, including plasma proteins and lipoproteins.

Activated endothelium produces cytokines such as IL-6, which is a potent regulator of acute phase response (73). The I/R-related acute inflammation boosts the host immune response by enhancing graft immunogenicity through the upregulation of MHC-II antigens (69, 74). Phenotypic change of activated EC has been well documented. Ischemia/reperfusion injury has a direct influence on the endothelial cell protective layer, known as glycocalyx. Experimental and clinical data reveal that I/R injury damages the glycocalyx, and particularly leads to shedding of the glycocalyx layer (64, 75, 76).
1.6.1.1 Shedding of the glycocalyx

The glycocalyx is a negatively charged mesh at the luminal side of vessel endothelium. It serves as a barrier to transvascular exchange of macromolecules and prevents cell adhesion to the endothelium. Several in vivo studies have revealed that the thickness of glycocalyx can reach up to 0.5 µm in the muscle capillaries (77). More recent studies indicate that the thickness of the glycocalyx increases with vascular diameter, at least in the arterial system, ranging from 2 to 3 µm in the small arteries (78) to 4.5 µm in the carotid arteries (79). The anionic polysaccharide structure of glycocalyx is comprised of glycoproteins, proteoglycans, and glycosaminoglycans (GAG) (Figure 5). Five structurally distinct GAG families exist: heparan sulfate (HS), chondroitin sulfate, dermatan sulfate, keratan sulfate, and hyaluronan. Heparan sulfate is the principal GAG of the endothelial glycocalyx. The major proteins are trans-membrane syndecan, which is comprised of four members (syndecan 1-4) and glycosylphosphatidylinositol (GPI)-anchored glypicans, with six members (glypican 1-6). Both syndecan and glypican contain attached HS and chondroitin sulfate.

There is ample of evidence available both in vitro and in vivo that the glycocalyx undergoes shedding in I/R injury. The early evidence of the effect of I/R injury on the glycocalyx has been demonstrated using isolated rat heart (80). The conclusion of the study was that the onset of reperfusion injury caused the disruption of the ultrastructure of the glycocalyx, probably through hydroxyl radicals generated from the Fenton reaction (80). Recently, Chappell et al. (81) demonstrated that I/R injury led to the shedding of syndecan-1, heparan sulfate proteoglycan (HSPG) and hyaluronan. More recently, the shedding of the endothelial glycocalyx during I/R injury has been demonstrated in humans (64). It has been also shown that rapid shedding of endothelial cell HSPG occurs after treatment by anti-endothelial cell antibodies and complement (82). Using a rat model of I/R injury and inflammation of mesentry post-capillary venules, Mulivor et al. (83) showed rapid shedding of venular glycocalyx. Moreover, an inflammatory stimulus, TNFα, caused the release of components of glycocalyx in isolated guinea pig hearts (84). It has been shown that syndecan-1 and 4 ectodomains are shed after treatment by thrombin and epidermal growth factor (85). However, treatment with heparinase selectively shed heparan sulfate, but not the syndecans (86). Taken together, the glycocalyx is shed after triggering multiple intracellular signaling pathways, which are activated by various physiological effectors. These signaling pathways involve protein kinase C (PKC), G protein couple receptors (GPCR), protein tyrosine kinase (PTK) and tissue inhibitor of the metalloproteinase-3 sensitive pathways (85, 87). Moreover, the shedding may be mediated by intracellular and/or membrane bound proteases or lyases activated or released by G protein signaling.
Figure 5. Components of the endothelial glycocalyx. Proteoglycans with long unbranched glycosaminoglycan (GAG) side-chains and glycoproteins with short branched carbohydrate side-chains are present in the cell membrane. The most abundant proteoglycans are heparan sulfate proteoglycans (HSPG). The members of HSPG family are syndecan and glypican. Syndecan is a transmembrane protein, which bears heparan sulfate chains distal from the plasma membrane. Glypicans are glycosylphosphatidylinositol (GPI)-anchored proteoglycans in the outer leaflet of the plasma membrane.

1.6.1.2 Role of the glycocalyx in inflammation

It has been shown that adenoviral gene expression of syndecan-1 protects against exaggerated inflammation after myocardial infarction (MI), mainly by reducing transendothelial adhesion and migration of leukocytes. Targeted deletion of syndecan-1 augmented inflammation through increased monocyte chemoattractant protein-1 expression and increased activity of matrix metalloproteinase-2 and -9 (88). The glycocalyx has the ability to bind a variety of proteins specifically through the heparin-binding domain. Some of the important proteins are xanthine oxidase, superoxide dismutase, antithrombin III, apolipoproteins, selectins and chemokines. Treatment with a potent inflammatory cytokine TNFα resulted in profound reduction of the thickness of glycocalyx, leading to activation of monocytes and inhibited anticoagulatory properties in a human study (89). Recently, it has been demonstrated that the superoxide- and nitric oxide-derived mediators resulted in disruption of the glycocalyx, which led to accumulation of polymorphonuclear leukocytes (PMN) in hearts subjected to I/R injury (90).
The components of glycocalyx shed due to activation of endothelium potentially serve as endogenous ligand for PRR, particularly TLR. A consequence of the engagement of the TLR on immune competent cells, especially DC, is the activation of the immune response. It has been shown that HS (91) and hyaluronan (92) shed after the activation of EC serve as endogenous ligands for TLR.

1.7 Effect of ischemia/reperfusion injury on tolerance

1.7.1 Ischemia/reperfusion injury as a link between the innate and adaptive immunity

Both animal models and clinical studies of transplantation suggest that I/R injury can alter the graft function and survival. It has been demonstrated that the up-regulation of pro-inflammatory factors is independent of the adaptive immune system and occurs before T cell mediated immune activation after allotransplantation (93, 94). He et al. (93) showed that macrophage infiltration and up-regulation of multiple cytokines, chemokines, and chemokine receptors occurs within the first day after transplantation in allogeneic, alymphoid and syngeneic groups. In line with this finding, studies using graft recipients deficient in various genes, including chemokines, cytokines, and other immune-associated genes frequently show prolonged graft survival. Further, gene analysis revealed which genes are upregulated during the early phase associated with I/R injury and suggested a role of the innate immunity (94). These findings imply that the antigen-independent processes interact with the adaptive immune system to augment graft rejection. One of the key factors stimulating the innate immune system appears to be I/R injury. The potent acute inflammatory cytokine IL-6 is remarkably upregulated in I/R injury as a consequence of TLR dependent activation of DC (93). Moreover, IL-6 is secreted by activated EC (95). Consequently, IL-6 blocks the suppressive effect of regulatory T cells (Treg, CD4+CD25+) (96). Moreover, in the presence of IL-6 and TGFβ, T cells differentiate into IL-17 producing Th17 cells, preventing Treg generation (97). Hence, I/R injury appears to be an important link between the innate and adaptive immune system. Recently, Kang and Bluestone have suggested that minimization of I/R injury will be a key element of tolerance induction strategies (98).

1.7.2 Minimization of I/R injury during tolerance induction

It has been shown that the immunotoxin plus NF-kB inhibitor 15-deoxyspergualin has yielded stable tolerance in a non-human primates model (99). In this study, the early administration of deoxyspergualin suggests a clear limitation to inducing
transplantation tolerance in the presence of I/R injury. Thus, this study strongly suggests that the innate immune system is poised to defeat allograft tolerance induction, so effective blockade of innate immunity must be in place early, to enable development of a tolerogenic environment. Walker et al. (100) showed that the absence of MyD88, a key TLR signaling adaptor, abrogates the innate immune system and facilitates inducible allograft acceptance. It has been shown that TLR4 signaling is central to both signaling on both donor and recipient cells, contributing to alloimmune response after cold I/R injury (101). These data suggest that I/R injury boosts early immune response and abrogates tolerance induction. Therefore, curbing the I/R injury may facilitate the clinical tolerance induction protocols. Activation of graft EC due to I/R injury creates an inflammatory milieu and jeopardizes the success of transplantation tolerance. Therefore, strategies aimed to protect the graft endothelium appear to be a useful tool against I/R injury.

1.8 Protection of endothelial cells

Strategies to protect endothelium activated as a result of I/R injury have been demonstrated in both in vitro and in vivo experiments (102-105). Substances bearing the properties of EC protection act as a repair coat for the endothelium, which is protected during immune-mediated damage (103). Substances sharing the properties of EC protection have been described in different animal models and in vitro and these substances are commonly known as EC-protectants.

1.8.1 Dextran sulfate as EC-protectant

Among several substances, low molecular weight dextran sulfate (DXS MW 5000) has been used extensively as a potent EC protectant. Laumonier et al. (102-105) proposed that release of HSPG from the activated EC facilitates binding of DXS, which may functionally replace the shed HSPG on the EC surface and thereby acts as a repair coat. Moreover, DXS binds to pig aortic endothelial cells (PAEC) after being activated with human serum, but not to quiescent PAEC. Binding specificities have been observed by using fluorescence labeled DXS. Moreover, DXS binding was favored by shedding of the natural EC-protective layer of HSPG by pretreatment of the cells with heparinase I. The repair coat prevents further damage of the cells by reestablishing an anticoagulatory and anti-inflammatory surface, or possibly mimicking its natural glycolandscape (Figure 6).
Figure 6. Protection of graft endothelium. Perturbation of the endothelial cell protective layer due to I/R injury leads to activation of the innate immune system. Tissue damage is mediated by infiltration of immunocompetent cells and deposition of complement components. Protection of the endothelium with “EC protectant” such as low molecular weight dextran sulfate (DXS, MW 5000) restores the quiescent and non-dangerous state by minimizing immune activation and subsequent graft damage.

The EC-protective effects of DXS have been proven in a hamster-to-rat xenotransplantation model (103). In this model, DXS was injected into the recipient during the first 2 weeks post-transplantation, combined with continuous cyclosporin treatment. This treatment protocol led to accommodation and long-term survival of the hamster xenograft. Binding of DXS to activated endothelium was also reported in a rat model of thrombotic microangiopathy (TMA) in the kidney. In this model, biotin-DXS staining was detected only on the surface of glomerular endothelium with TMA, but not in the kidney without TMA (104). Another similarly acting substance used in experiments is multimeric tyrosine sulfate (102). Functional replacement by DXS on activated EC mediates several key protective functions including inhibition of complement activation, coagulation and cell infiltration.
1.8.1.1 Inhibition of complement activation and coagulation

Inhibition of the classical complement activation pathway by DXS is mediated through potentiation of C1-inhibitor (106). C1-inhibitor binds to the C1s molecules and dissociates them from C1q. Other than potentiation of C1-inhibitor, DXS also binds to exposed thioesters of activated C3 and C4 and thus inhibits their deposition (106). The exact mechanism leading to potentiation of C1-inhibitor by DXS is unknown (107); however, sulfate groups seem to play a role. Dextrin, an \( \alpha(1\rightarrow4) \) linked polyglucose similar to dextran, which is \( \alpha(1\rightarrow6) \) linked, has no anti-complement effect, whereas dextrin bearing carboxylic and benzyl amide sulfonate groups inhibits complement activation \textit{in vivo} and \textit{in vitro} in experimental models (107, 108). The interaction with the polyanion binding site on factor H has been proposed to be the mechanism by which DXS inhibits the alternative pathway of complement activation (109). DXS interacts with the factor H polyanion recognition site and by this enhances the binding of factor H to the C3b-target complex. The affinity of factor H to C3b is regulated at or near the 13\textsuperscript{th} short consensus repeat (SCR) domain of factor H, which is a positively charged site. \textit{In vitro} investigation of DXS in a model of antibody-mediated cytotoxicity revealed that deposition of C1q, C3b and C4b from human serum on PAEC was dose-dependently inhibited (103). In addition, it inhibits the coagulation system by enhancing the anticoagulatory activity of antithrombin III and C1-inhibitor against activated Factor XI (110). Moreover, DXS interferes with platelet adhesion (111). In a tubing loop model, DXS showed dose-dependent inhibition of instant blood mediated inflammatory reaction (IBMR), with an inhibition of complement activation and coagulation (112).

1.8.1.2 Effect on inflammatory cell infiltration

It has been shown that the addition of DXS to blood cardioplegia attenuates I/R injury in a model of porcine cardiopulmonary bypass. Moreover, the addition of DXS reduced neutrophil infiltration in porcine myocardium compared with the PBS control group (95). Dextran sulfate inhibits E-selectin mediated neutrophil adhesion to endotoxin activated human umbilical vein EC (113). In another study, although DXS showed lack of effect on neutrophil aggregation, it was found to inhibit N-formyl-methionyl-leucyl-phenylalanine (fMLP)-induced elastase release in a concentration dependent manner in neutrophils. (114). It has been also shown that adhesion of neutrophils was abolished by the treatment of the platelets with DXS (115). Neutrophils interact with platelets at the site of ischemic damage to promote thrombosis and vascular occlusion. Thus, the inhibitory capacity of DXS not only reduces the direct inflammatory effect mediated by neutrophils; it also helps to minimize thrombus formation. Studies of the effect of DXS on lymphocyte localization
in complement deficient mice revealed that DXS reduced cell localization without the help of terminal complement components (116).

1.8.1.3 Other immune regulatory effects of dextran sulfate

Recently, Spirig et al. (91) showed that DXS prevents TLR-mediated maturation of human monocytes derived DC, which were treated with various TLR ligands as well as HS as an endogenous ligand. Dendritic cells treated with DXS inhibited phosphorylation of IkappaB-α and activation of NF-kappaB, suggesting that DXS impeded the link between innate and adaptive immunity (91). IFN-γ-activated EC actively contribute in initiating immune responses by interacting with the immune system via MHC-II proteins. It has been shown that DXS blocks IFN-γ-induced surface expression of HLA-DR molecules by preventing transcription of the gene encoding CIITA, a transactivator protein required for IFN-γ-inducible expression of MHC-II gene (117). Moreover, IFN-γ-induced phosphorylation of Stat1 and Jak2 is also inhibited by DXS (118). Oxygen derived free radicals are the most potent factor mediating detrimental effects in I/R injury. It has been demonstrated that DXS protects rat coronary and porcine aortic endothelial cells from oxygen derived free radical injury, suggesting an anti-oxidative property of DXS (119). More recently, it has been demonstrated that DXS attenuated proliferation of human airway smooth muscle cells (ASM) and IL-13-induced eotaxin (CCL11) released from ASM (120, 121).

1.9 Role of hepcidin in anemia associated with surgical procedures

Surgical procedures inevitably associated with organ transplantation induce a post-operative acute phase reaction (122), which in turn affects a number of homeostasis mechanisms including iron homeostasis, amongst others. The outcome is commonly referred as anemia of inflammation.

Recently, an increase in an acute phase protein known as hepcidin, which is produced in the liver and has an antimicrobial activity, was shown in patients with anemia of inflammation (123). Knocking out the hepcidin gene in mice (Usf2 +/- mice) leads to severe iron overload, suggesting that hepcidin plays an important role in iron metabolism (124). Conversely, transgenic mice expressing hepcidin constitutively die at birth with severe iron deficiency (125).
The human hepcidin molecule consists of 25 amino acids and negatively regulates iron uptake from the intestine (126), release from the hepatic store and recycling by macrophages (127), which are the three main gateways of maintaining the body iron homeostasis. Hepcidin synthesis is greatly increased within one day after an iron rich diet in mice and in humans (128). However, the mechanism of iron regulation remains elusive. Iron absorption from the intestine and release from the body iron store are greatly enhanced by the erythropoietic stimuli associated with blood loss and anemia, whereas hepcidin gene expression is markedly down regulated in blood loss and hypoxia (129), which may account for the increased iron absorption and iron release from the reticulo-endothelial system. Thus, the serum hepcidin level is markedly reduced during erythropoietic stimuli and increased during infection independent of blood loss or hypoxia (130). It has been shown that IL-6 is necessary and sufficient for the upregulation of hepcidin in inflammation (128). Anemia of inflammation is associated with chronic and acute infection, generalized inflammatory disorders and malignancy. Cytokines, particularly IL-6, induced during inflammation upregulate hepcidin. Subsequently, the iron supply is limited in bone marrow for erythropoiesis.

Although the role of hepcidin in iron homeostasis has been extensively considered, determining reliable methods for measuring serum hepcidin concentration have been problematic. There is no standard method available to quantify serum hepcidin in clinical settings. Hence, quantification of hepcidin is a challenging task for understanding the clinical pathogenesis of hepcidin regulated ion homeostasis.
2 Results

2.1 Paper I – Dextran Sulfate Facilitates Anti-CD4 mAb-induced Long-Term Rat Cardiac Allograft Tolerance After Prolonged Cold Ischemia

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Ischemia/reperfusion (I/R) injury leads to activation of the innate immune response of the recipient. We hypothesized that this boost of immunogenicity is the reason for failure of immunological tolerance induction in the context of prolonged graft ischemia time. Here we show that treatment with the endothelial cell (EC) protectant, dextran sulfate (DXS, MW 5000) restores a quiescent and 'non-dangerous' state of the graft. This facilitates transplantation tolerance, induced by non-depleting anti-CD4 mAb (RIB 5/2).

\textbf{Dextran sulfate facilitates anti-CD4 mAb-induced tolerance.} Ischemia/reperfusion-induced endothelial activation and the subsequent innate immune responses are attenuated by DXS treatment by establishing “non-dangerous” endothelium. Consequently, this strategy facilitated the tolerance induction capacity of anti-CD4 mAb.
Dextran Sulfate Facilitates Anti-CD4 mAb-Induced Long-Term Rat Cardiac Allograft Survival After Prolonged Cold Ischemia

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Introduction

Induction of donor-specific transplant tolerance appears to be a potential alternative in clinical transplantation to solve the major drawbacks associated with current immunosuppressive therapies. However, attempts to transfer immunological tolerance induction strategies from experimental models to clinical settings have as yet not been very successful. In clinical organ transplantation, the establishment of tolerance is hampered by problems such as ischemia/reperfusion (I/R) injury and brain death of the organ donor. There is considerable evidence supporting the fact that I/R injury influences tolerance induction (1). Consequently, it was recently proposed that minimization of I/R injury will be a key element in tolerance induction strategies (2).

I/R injury induces an inflammatory reaction, which promotes chronic rejection and jeopardizes allograft survival (3,4). The I/R-related acute inflammation leads on one hand to acute organ damage and on the other hand boosts the host immune response by enhancing graft immunogenicity through upregulation of MHC class II antigens, ICAM-1, P- and E-selectin, as well as costimulatory molecules, particularly CD80 and CD86 (5–7). As an additional pro-inflammatory mediator also, the activation of complement plays a major role in I/R injury. While the involvement of the classical and alternative complement pathways in I/R injury is generally accepted (8–10), growing evidence suggests that also neopterin responds on EC and recognized by pattern recognition molecules such as mannose-binding lectin (MBL), either directly or via binding of naturally occurring IgM, triggering complement activation and subsequent graft damage (11,12). I/R injury-related enhancement of immune responses is in part also mediated by signaling via Toll-like receptors (TLR) (13). Moreover, soluble heparan sulfate proteoglycans (HSPG), shed from the endothelial glycoprolyx due to I/R injury-induced EC activation, provoke TLR4-mediated dendritic cell activation and thereby link innate and adaptive immunity (14–16). Taken together, the activation of graft EC plays a major role in I/R-mediated regulation of the innate immune response and thus the outcome of graft survival.

Ischemia/reperfusion injury leads to activation of graft endothelial cells (EC), boosting antigrant immunity and impeding tolerance induction. We hypothesized that the complement inhibitor and EC-protectant dextran sulfate (DXS, MW 5000) facilitates long-term graft survival induced by non-depleting anti-CD4 mAb (RIB 5/2). Hearts from DA donor rats were heterotopically transplanted into Lewis recipients treated with RIB 5/2 (20 mg/kg, days—1,0,1,2,3; i.p.) with or without DXS (grafts perfused with 25 mg, recipients treated i.v. with 25 mg/kg on days 1,3 and 12.5 mg/kg on days 5,7,9,11,13,15). Cold graft ischemia time was 20 min or 12 h. Median survival time (MST) was comparable between RIB 5/2 and RIB 5/2+DXS-treated recipients in the 20-min group with >175-day graft survival. In the 12-h group RIB 5/2 only led to chronic rejection (MST = 49.5 days) with elevated alloantibody response, whereas RIB 5/2+DXS induced long-term survival (MST >100 days, p < 0.05) with upregulation of genes related to transplantation tolerance. Analysis of the 12-h group treated with RIB 5/2+DXS at 1 day posttransplantation revealed reduced EC activation, complement deposition and inflammatory cell infiltration. In summary, DXS attenuates I/R-induced acute graft injury and facilitates long-term survival in this clinically relevant transplant model.

Key words: Anti-CD4 mAB, cold ischemia, complement, dextran sulfate, long-term graft survival, rat heart transplantation

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have been shown to provide beneficial effects in hamster-to-heat and pig-to-human xenotransplantation models (17, 18). Banz et al. demonstrated that protection of the cardiatic endothelium using DXS mitigates inflammation in a closed chest porcine model of acute myocardial infarction (19). Treatment with DXS downregulated tumour necrosis factor and prevented inflammation in this model. The beneficial effect of DXS therefore attenuated in myocardial infarction make this substance a possible candidate for use in a setting of transplantation tolerance. In fact, strategies to inhibit innate immunity early after transplantation have previously been successful in experimental models. Use of the NF-κB inhibitor 15-deoxy-atrianaphthoquinone induced allograft tolerance in an anti-CD3-directed xenogeneic model (20), and lack of MyD88 signaling induced long-term allograft survival in a mouse model (21), supporting the idea that inhibition of innate immunity provides a clinically relevant strategy to facilitate transplantation tolerance. We have, therefore, used a combined approach of attenuating IR injury-induced innate immune response by DXS and non-depleting anti-CD4 mAb treatment (22) in order to facilitate long-term graft survival in a rat cardiac allotransplantation model with prolonged ischemia time.

**Materials and Methods**

**Animals and cardiac transplantation**

Inbred male DA (RT11)2 and Lewis (RT11)1 rats, 8–12 weeks of age, were purchased from Harlan Nederland B. V. (Horst, The Netherlands). Heterotopic heart transplantation from DA (donor) to Lewis (recipient) was carried out using a modification of the original technique described by Oto and Lindsey (23). The procured heart was kept for 20 min or 12 h on ice in Cellstar (IMTM Syperm, Lyon, France) before transplantation. Time of reoxygenation was defined as the day of complete reperfusion of heart. All experiments in this study were performed according to current versions of Swiss Law on Animal Protection.

**Anti-CD4 mAb and DXS treatment protocol**

The anti-CD4 mAb RII 52 was developed at the University of Rostock, Germany (22) and produced in vitro by EXBIO Peha (Prague, Czech Republic). Recipient rats were treated i.p. with RII 52 at 20 mg/kg body weight on days 1–5, 1–2, and 3 to induce long-term graft acceptance. DXS (MW, 3000) was purchased from Fuka (Buchs, Switzerland). Immediately before transplantation, the grafts were perfused with 25 mg of DXS dissolved in 2 mL of sterile PBS followed by perfusion with PBS to remove unbound DXS. After transplantation, the recipients were treated with DXS at 25 mg/kg body weight on days 1 and 3 and then 12.5 mg/kg every other day until day 15.

**Quantitative RT-PCR**

Total RNA from homogenized tissue was isolated using the Miniprep Kit (Stratagene, Heidelberg, Germany) and reverse transcribed into cDNA using the murine leukemia virus reverse transcriptase (Gibco BRL, Gaithersburg, MD). Samples were analyzed using qRT-PCR as previously described (24). Reactions were run using the Model 7700 Sequence Detector (TaqManTM, Applied Biosystems, Foster City, CA). β-actin was used as a housekeeping gene for the samples. The sequences of the oligonucleotides and panels used in qRT-PCR were either described previously (25) or displayed in Table 1. Sense and antisense oligonucleotides were purchased from Metabioc (Munich, Germany). Primers were obtained from Eurogentec (Cologne, Germany).

**Detection of allotypic antibody response**

To analyze donor-specific allospecific responses sera were collected from recipients at days 5, 7, 11, 19, and 100. Aliquots containing 1 × 10⁶ spleenocytes from DA rats (donor strain) were incubated 45 min at 4°C with diluted recipient serum (1:4). The washed cells were then incubated 30 min at 4°C with FITC-conjugated goat anti-rat IgG (Serotec, Westcliff, Germany) and PE-conjugated goat anti-rat IgM (Jackson ImmunoResearch, Suffolk, UK). Cells were analyzed with a BD FACScan flow cytometer and the data quantified using FlowJo software (Tree Star Inc., Ashland, OR). The levels were expressed as median channel fluorescence.

**Histology and immunohistochemistry**

Tissue samples from the grafts were preserved in 4% buffered formaldehyde and stained with hematoxylin and eosin (H&E), Elastica van Gieson (EVG) and Masson’s Trichrome (MT). For immunohistological analysis, cryosections (5 μm) were stained with mouse anti-rat mAbs against glomerulosclerosis (GBS4), morphoexines/morphoexines (ED1), CD4 (W3/25), CD8 (CD8-B; all from Serotec, and T cells [15-16-1A, Abcam, Cambridge, UK]. Positive staining was detected using the Envision kit (DAKO). Twenty high-power field images were evaluated per section and graded by eye on a 0 to 4 scale (0 = not detectable, 1 = mild; 2 = moderate; 3 = strong, 4 = very strong) by a researcher blinded to the study.

**Immunofluorescence staining**

Atoine-fixed cryosections were used for immunofluorescence staining. The primary antibodies used were mouse monoclonal anti-human heparan sulfate (Seikagaku, Tokyo, Japan), rabbit anti-human VWF and rabbit anti-human CD34 (DAKO), mouse monoclonal anti-CD201 (Serotec) and rabbit anti-rat CD3 (kindly provided by Prof. P Morgan, University of Cardiff, UK). All human-specific primary antibodies were cross-reactive with the respective rat antigens. Secondary antibodies were goat anti-rabbit IgG (FITC; Southern Biotechnology Associated, Birmingham, AL), goat anti-mouse IgM (FITC).

Table 1: Oligonucleotides and panels used to quantify mRNA expression

<table>
<thead>
<tr>
<th>Gene</th>
<th>qRT-PCR primers/panel (assay no.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bag-1</td>
<td>5′-CATGACCACCCAGAGAGTCTG-3′&lt;br&gt;5′-TTCTGAATCCACAGGAGGTTTCTTCCG-3′&lt;br&gt;probe 5′-TTCATAGGACCCAGGATTGTTTCCCTC-3′&lt;br&gt;rev 5′-CAAAGGGTCTGCGGAGGTTTCCCTC-3′&lt;br&gt;CD52</td>
</tr>
</tbody>
</table>

CD40L Rn01184362_m1 (Applied Biosystems)<br>CTLA4 Rn01565456_m1 (Applied Biosystems)
Dextran Sulfate Facilitates Long-Term Allograft Survival

In the experimental groups with 20 min of cold graft ischemia, treatment with RIB 5/2 mAb only or in combination with DXS significantly prolonged allograft survival as compared with the PBS-treated vehicle control group (p < 0.001). MST of the RIB 5/2 only and RIB 5/2+DXS groups were 86 and 81 days, respectively (Table 2). In both groups 28% of the grafts survived more than 175 days. To determine the effect of DXS, graft survival was monitored after treatment with DXS only and compared with the PBS-treated control group. MST of these groups were 7.0 and 5.5 days, respectively. Although DXS treatment prolonged the graft survival compared with PBS controls, analysis of the grafts in both groups revealed signs of severe acute rejection (results not shown).

To be closer to the clinical situation of deceased heart donors, long-term survival induced by RIB 5/2 mAb treatment, with or without additional treatment by DXS, was challenged by prolonged graft ischemia time. After 12 h of cold ischemia, grafts were transplanted and the recipients received RIB 5/2 alone or in combination with DXS. Treatment with RIB 5/2 only was no longer sufficient for maintaining the long-term graft survival and all grafts were rejected with a MST of 49.5 days (Figure 1). Moreover, with RIB 5/2 only, the MST was significantly reduced in the 12-h ischemia group as compared with 20-min ischemia (p < 0.001). As compared with RIB 5/2 only, graft survival was significantly increased in RIB 5/2+DXS-treated recipients (MST > 100 days, p < 0.05). In this group, more than 57% of the grafts survived >100 days. In another, separate series of experiments, the same protocol was repeated and survival was monitored up to 40 days. In this study, the graft survival percentage was 100% and 66% at 40 days in the RIB 5/2+DXS and RIB 5/2 only-treated groups, respectively (n = 6 per group). There was no significant difference in MST between 20-min and 12-h ischemia groups treated with RIB 5/2+DXS (p = 0.858).

Histology

Histologies of long-term surviving and chronically rejected grafts were compared with naive hearts (Figure 2A). Chronically rejected grafts in both groups with 20 min of ischemia showed cell infiltration, intimal thickening and fibrosis (Figure 2B). Histological analysis of long-term surviving grafts showed no evidence of cellular infiltration, parenchymal fibrosis or intimal thickening in either group (Figure 2C). Grafts harvested after chronic rejection (MST = 49.5 days) from recipients treated with RIB 5/2 only in the 12-h ischemia group showed characteristic signs of chronic rejection. Severe cellular infiltrations were observed in all these grafts by H&E staining (Figure 2D). Moreover, EVG and MT stainings revealed marked intimal thickening and parenchymal fibrosis, respectively. In contrast, long-term surviving grafts, from recipients treated with RIB 5/2+DXS, did not show detectable cellular infiltration, intimal thickening or parenchymal fibrosis (Figure 2E). Histology of grafts was always assessed by a blinded pathologist. Statistical analysis showed correlation with RIB 5/2 and DXS treatment. The results are expressed as mean ± SD. Survival of the allografts was examined using Kaplan–Meier analysis and groups were compared using the log-rank test. Data were analyzed using Student’s t-test. For gene expression analysis we used Mann-Whitney U-test. Significance was determined with p < 0.05.

Table 2: Survival of grafts after 20 min of cold ischemia in recipients treated with PBS, DXS and RIB 5/2 alone or in combination with DXS

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Graft survival (day)</th>
<th>Median survival time</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS (control)</td>
<td>4</td>
<td>5, 6</td>
<td>5.5</td>
</tr>
<tr>
<td>DXS</td>
<td>6</td>
<td>7, 7, 7, 8</td>
<td>7.0</td>
</tr>
<tr>
<td>RIB 5/2</td>
<td>7</td>
<td>74, 74, 84, 96, 113, &gt;175, &gt;175</td>
<td>96.0</td>
</tr>
<tr>
<td>RIB 5/2+DXS</td>
<td>7</td>
<td>69, 72, 86, 107, &gt;175, &gt;175</td>
<td>81.0</td>
</tr>
</tbody>
</table>

Note: MST = Median Survival Time

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Figure 1: DXS facilitates RIB 5/2 induced long-term graft survival after 12 h of cold graft ischemia time. DA-to-Lewis heterotopic rat cardiac allotransplantation was performed with 12 h of cold graft ischemia. Recipients were treated with either RIB 5/2 only or RIB 5/2+DXS. Kaplan–Meier graft survival curves are shown for both treatment groups. *p < 0.05 as compared with RIB 6/2 only.
Expression of gene markers
Chronically rejected (RIB 5/2 only, at time of rejection) and long-term surviving allografts (RIB 5/2+DXS, 100 days), both with 12-h cold ischemia, were analyzed for markers described in the context of allograft tolerance as well as rejection. In addition, grafts of both groups were analyzed at 40 days. The changes in gene expression, indicated as “fold changes” in the RIB 5/2+DXS versus the RIB 5/2 only-treated groups, are shown in Table 3. Most of the observed changes did not reach statistical significance. However, the recently described gene TOAG-1, associated with long-term graft acceptance (26), was upregulated 2.7-fold ($p = 0.031, n = 5$) and 82.9-fold ($p = 0.028, n = 4$) in allografts treated with RIB 5/2+DXS at 40 days and 100 days, respectively (Table 3). The other significantly upregulated genes were CD25 ($p = 0.030$), CD3 ($p = 0.031$) and CD40 L ($p = 0.031$), all at 40 days.

Humoral immune response
Serum IgM levels against donor splenocytes showed a time-dependent increase in the RIB 5/2 group with 12 h of cold ischemia, whereas they remained more or less stable in the respective RIB 5/2+DXS group (Figure 3). Similarly, antidonor IgG levels did not change in the RIB 5/2+DXS group, whereas a steep increase was seen in RIB 5/2-only treated recipients that peaked at day 7, posttransplantation and then gradually declined with time.

Effect of prolonged ischemia time on early posttransplantation changes
Grafts subjected to 20-min or 12-h cold ischemia were analyzed histologically 1 day after transplantation. Only minimal histological changes were observed in the 20-min ischemia group treated with either PBS, RIB 5/2 or RIB
Dextran Sulfate Facilitates Long-Term Allograft Survival

Table 3: Allograft gene expression in RIB 5/2+DXS-treated and RIB 5/2 only-treated groups with 12-h cold ischemia at 40 and 100 days posttransplantation

<table>
<thead>
<tr>
<th>Gene</th>
<th>Category</th>
<th>RIB 5/2+DXS/RIB 5/2 only (40 day post tx)</th>
<th>RIB 5/2+DXS/RIB 5/2 only (40.5 day post tx)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(Fold change, mean, n = 5)</td>
<td>(Fold change, mean, n = 4)</td>
</tr>
<tr>
<td>α-1,2-mannosidase</td>
<td>N-Glycosylation</td>
<td>+1.4</td>
<td>+1.0</td>
</tr>
<tr>
<td>Bag-1</td>
<td>Cytoprotection</td>
<td>+1.2</td>
<td>+2.4</td>
</tr>
<tr>
<td>CD25</td>
<td>Activated T-cell related</td>
<td>+2.8*</td>
<td>+1.3</td>
</tr>
<tr>
<td>CD3</td>
<td>Costimulation</td>
<td>+3.0*</td>
<td>+1.8</td>
</tr>
<tr>
<td>CD40L</td>
<td>Costimulation</td>
<td>+3.9*</td>
<td>+2.8</td>
</tr>
<tr>
<td>CTLA4 (CD152)</td>
<td>Costimulation</td>
<td>+2.3</td>
<td>+1.6</td>
</tr>
<tr>
<td>Foxp3</td>
<td>Regulatory T-cell related</td>
<td>+3.5</td>
<td>+2.9</td>
</tr>
<tr>
<td>HO-1</td>
<td>Cytoprotection</td>
<td>+1.1</td>
<td>+6.7</td>
</tr>
<tr>
<td>IL-17</td>
<td>Cytokines</td>
<td>+2.7</td>
<td>+2.1</td>
</tr>
<tr>
<td>Perforin</td>
<td>Cytotoxic T-cell related</td>
<td>+1.7</td>
<td>+1.2</td>
</tr>
<tr>
<td>RORγt</td>
<td>Th17-cell related</td>
<td>+3.9</td>
<td>+3.5</td>
</tr>
<tr>
<td>TOAG-1</td>
<td>Unknown</td>
<td>+2.7*</td>
<td>+62.9*</td>
</tr>
</tbody>
</table>

*p < 0.05.

5/2+DXS. These changes were characterized by mild cellular infiltration and perivascular edema, and were comparable among all the treatment groups (Figures 4A, C, E, G). In the PBS-treated control group with 12 h of graft ischemia time, we observed severe cellular infiltrations, edema, hemorrhages and myocardial necrosis (Figure 4B). RIB 5/2-only treatment reduced the severity of these histological changes, but a marked ongoing inflammation, characterized by cellular infiltration, edema and hemorrhages, was clearly observed in all grafts (Figure 4D). Strikingly, treatment by RIB 5/2+DXS as well as DXS alone reduced the inflammatory changes as compared with RIB 5/2 only (Figure 4F, H). DXS treatment thus clearly reduced early graft injury, i.e. cellular infiltration, edema and other inflammatory changes in the 12-h graft ischemia group.

**DXS attenuates infiltration of mononuclear cells**

Since histological analysis revealed significant reduction of graft inflammatory cell infiltration in RIB 5/2+DXS treatment compared with RIB 5/2 only, we analyzed immunohistochemically the type of infiltrating cells. As shown in Figure 5, RIB 5/2+DXS or DXS-only treatment led to a significant reduction of both granulocyte (HIS48) and monocytes/macrophages (ED1) infiltration in the graft tissue. Quantitative analysis of cell infiltration revealed that the numbers of ED1- and HIS48-positive cells were significantly reduced in grafts from RIB 5/2+DXS-treated recipients compared with RIB 5/2 only-treatment (Figure 5B, p < 0.01). Only minimal infiltration by T cells (15–18A1) was observed with no difference between the two groups, and also the detection of CD4+ (W3/25) and CD8+ T cells (OX-8) revealed only minimal staining with no apparent difference between the RIB 5/2 only and RIB 5/2+DXS groups (not shown).

**Activation and damage of graft endothelial cells**

Graft EC were assessed 1-day posttransplantation in the 12-h ischemia groups. Staining for vWF and CD31 was circumferentially detected in blood vessels of grafts of RIB 5/2+DXS-treated recipients and this was comparable with the naive heart of these animals (Figure 6A). In contrast, remarkably diminished stainings for vWF and CD31 were

![Figure 3: Humoral immune response. Sera from RIB 5/2 only and RIB 5/2+DXS-treated groups were collected at days 0, 3, 7, 14, 28, and 100. Splenocytes from DA rats were incubated with the sera at 1/4 dilution and stained with FITC-conjugated anti-rat IgG and PE conjugated anti-rat IgM. Flow cytometric analysis of serum IgG and IgM antibody levels are shown. Data are represented as mean ± SD (n = 4).](image-url)

observed in allografts from RIB 5/2 only-treated recipients. As compared with the naïve hearts, staining for heparan sulfate (HS) was markedly reduced in the RIB 5/2 only-treated group (Figure 6B). Interestingly, quantitative analysis of HS in RIB 5/2=DXS-treated grafts revealed higher fluorescence intensities than the naïve hearts. This may be due to binding of DXS to the endothelium and subsequent cross-reaction with anti-HS antibody. In fact, cross-reactivity of the used anti-HS antibody with DXS was confirmed in a competitive inhibition experiment (data not shown).

**Binding of DXS-Fluo to the allograft vasculature**
The grafts subjected to 20 min or 12-h ischemia were perfused with 10 mg of DXS-Fluo in 2 mL of sterile PBS followed by perfusion with PBS in order to remove unbound

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Figure 5: DXS inhibits infiltration of granulocytes and monocytes/macrophages. Grafts subjected to 12-h cold ischemia were analyzed 1 day posttransplantation. (A) Immunohistochemical staining for granulocytes (HIS48), monocytes/macrophages (ED1) and T cells (16–16A1). Results are representative of three independent experiments, magnification: ×150. (B) Quantitative analysis of the positive staining (20 high-power fields scored by eye by an independent researcher blinded to the study). Data are represented as mean ± SD (**p < 0.01; n = 3).

substance. Analysis of these grafts revealed strong binding of DXS-Fluo only in the 12-h ischemic grafts and there was no binding to grafts after only 20 min of cold ischemia (Figure 7). Binding of DXS-Fluo was completely blocked after the prior perfusion of the graft with unlabelled DXS (data not shown). In addition, DXS was administered in vivo at 25 mg/kg i.v. 1 day after transplantation, followed by immediate graftectomy and analysis for binding of DXS-Fluo. Also, in these in vivo experiments, binding of DXS-Fluo was only observed in grafts subjected to 12 h of cold ischemia. Moreover, no binding of DXS-Fluo was detected in the native heart, liver, kidney, lung and spleen (data not shown).

DXS specifically attenuates deposition of complement
Deposition of C3b/c and C9 was detected in grafts of RIB 5/2-treated recipients and significantly reduced by additional DXS treatment in the 12-h ischemia group (Figure 8A). In addition, the effect of DXS on systemic complement activation was analyzed. Lewis rats were treated with a single dose of 25 mg/kg of DXS i.v. without receiving a transplant, and their serum analyzed for classical pathway complement activity by a CH50 test. Six hours after injection of DXS the CH50 values were only 11% lower than the recorded baseline value (Figure 8C: p = 0.684; 6 h vs. baseline). Also, long-term monitoring up to 5 days during...
ongoing DXS treatment in transplant recipients revealed no reduction of CH50 values (Figure 8D), suggesting that the used dosage and application scheme of DXS had no significant influence on systemic complement activity.

**Effect of DXS on coagulation system**

The effect of DXS on the coagulation system was monitored after administration of a single dose of DXS at 25 mg/kg i.v. The aPTT values rose to >600 s for up to 90 min after DXS administration and then quickly dropped again. After 360 min the values were back to nearly baseline level (Figure 9A; n = 3). Therefore, systemic administration of DXS led to a transient inhibition of the coagulation system. In contrast, we did not observe an effect of DXS on platelet counts. Platelet counts changed from 544 ± 32 x 10^6 cells/µL at baseline to 600 ± 64 x 10^6 cells/µL 1 day after DXS injection (Figure 9B; p = 0.269, n = 3).

**Discussion**

Early inflammatory changes associated with brain death and prolonged cold ischemia, critically influence the function and survival of allografts [27-29]. Indeed, strategies aimed at prevention of graft I/R injury were shown to promote long-term allograft acceptance [20,21]. In this study, a new therapeutic approach targeting innate immunity by using low molecular weight dextran sulfate in combination with the nondepleting anti-CD4 mAb RIB 5/2, significantly

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Figure 7: Binding of fluorescence-labeled DXS (DXS-Fluo) to allografts. Donor hearts were subjected to 20-min or 12-h cold ischemia in Celsior solution followed by perfusion with 10 mg/mL of DXS-Fluo (2 mL). Tissues were embedded in OCT, snap-frozen, sectioned and analyzed. (A) DXS-fluo binding to the graft subjected to 20 min and (B) 12 h of cold ischemia. One representative example of three independent experiments is shown, magnification: x400.

Figure 8: Effect of DXS treatment on complement deposition. (A) DA hearts subjected to 12 h of cold ischemia were transplanted into Lewis rats, which received RIB 5/2 with or without DXS. Recipients were sacrificed at day 1 after transplantation and the grafts immunostained for C3b/c and C9. (B) Quantitative analysis of deposition of complement by Image-J software. The shown sections are representative of three analyzed grafts from each group, magnification: x400. Complement activity in serum was monitored by CH50 assay after (C) a single dose of i.v. injection of DXS (25 mg/kg) or (D) continuous treatment until day 5.

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extended the survival of allografts transplanted after prolonged cold ischemia.

As expected, RIB 5/2 treatment was able to prolong graft survival in recipients of cardiac allografts, which were subjected to only 20 min of cold ischemia. However, the same treatment led to chronic rejection, characterized by severe inflammatory cell infiltration, intimal thickening and fibrosis, which are typical features of cardiac allograft vasculopathy (CAV). If the cold ischemia time of the grafts was extended to the clinically more relevant 12 h. This picture of CAV could be prevented and long-term graft survival reestablished if RIB 5/2 treatment was complemented by i.v. injection of DKS for 2 weeks posttransplantation. Intragraft gene expression analysis at 40 days and 100 days revealed a significant upregulation of the gene TOAG-1 at both time points, consistent with the recent finding that this gene is specifically and reproducibly upregulated in long-term surviving allografts (25). The observed upregulation of genes such as CD25, CD3 and CD40 L in long-term surviving allografts may be linked to the fact that long-term graft acceptance is a result of a complex and dynamic interplay between regulatory and effector T cells. Pro-inflammatory factors were described to be upregulated in long-term surviving grafts compared with rejected grafts and to play a dominant role in maintaining tolerance (30,31). Moreover, elevation of pro-inflammatory related genes does not necessarily reflect the rejection rate (30,32).

In our experiments, chronic graft rejection was linked to a pronounced IgM and IgG alloantibody response, which was absent in RIB 5/2+DKS-treated recipients with long-term surviving allografts. This finding is in line with recent reports (31), and we conclude that inhibition of the early innate immune response by DKS treatment may play an important role in lowering the antiallograft IgG and IgM response thus prevent chronic rejection.

We showed that prolonged cold graft ischemia for 12 h, but not 20 min ischemia, critically influenced damage and activation of the graft endothelium characterized by the shedding of HSPG. As a consequence, the graft endothelium seems to become a target for binding of DKS, which then reestablishes its anti-inflammatory and anticoagulatory properties. In other words, DKS reinstates the "non-dangerous" (33) properties of the graft endothelium. Using a fluorescence-labeled variant of DKS, DKS-Fluo, we could indeed observe binding of DKS to graft endothelium after 12 h of cold ischemia, but not after 20 min. As shown earlier by ourselves and others (34,35), binding of DKS was inversely correlated to the endothelial expression of HSPG.

Activation of the complement system and subsequent complement-mediated tissue damage is a key feature of IR injury. While DKS had almost no systemic effect on the complement system in our experiments, it significantly reduced local complement deposition at 1-day posttransplantation. In line with reports, which highlight the importance of local complement production (36), our results indicate that local inhibition of complement activation may protect the allograft during the tolerance induction phase and therefore facilitate the latter. Moreover, early graft infiltration by granulocytes and monocytes/macrophages critically influences the long-term allograft response (37,39). Therefore, it is tenable that the observed inhibition of granulocyte and monocytes/macrophages infiltration by DKS is positively correlated with long-term graft survival. Matsumiya et al. showed that DKS inhibits E-selectin-mediated neutrophil adhesion to activated EC (39). Furthermore, DKS may attenuate anaphylatoxin-mediated recruitment of inflammatory cells through inhibiting complement. Consistent with this observation, it was also suggested that DKS has immunosuppressive properties. DKS was shown to inhibit the IFN-γ-induced MHC class II expression on
endothelial cells (40). Although soluble DXS serves as a
competitive inhibitor of the binding of IFN-γ to membrane
IFN-γR1s, immobilized DXS can bind with IFN-γ (41), sug-
gest that DXS has the capacity to bind immunoreg-
ulatory elements and subsequently target them to acti-
vated graft endothelium. However, the relative importance
of DXS-mediated binding of immunoregulatory elements
to the EC surface remains to be determined in separate
experiments.

As expected by the fact that DXS is an anticoagulatory sub-
stance (42), it has a transient effect on coagulation in the
untreated graft recipients. However, administration of the
indicated dose of DXS in our model did not cause any major
bleeding complications. It is as yet unclear whether or not
this anticoagulatory effect is beneficial for long-term graft
acceptance. In view of a clinical application of DXS or sim-
ilarly acting substances, the anticoagulatory effect needs
to be considered, and further investigations on the need of
anticoagulation to achieve the DXS-mediated attenuation
of innate immunity are warranted. Generation of antide-
xtran sulfate antibodies, which might limit the clinical use
of DXS, has not been described following i.v. administration
so far, despite of quite extensive use of DXS in different
animal models as well as pre-clinical studies (17,43,44).

It has been reported that the use of FY720 in combination
with RIB 592 prevents tolerance induction (45). Therefore,
the selection of drugs for synergistic treatment with tol-
erance induction protocols appears to be a challenge. Our
novel approach of graft-targeted treatment by DXS might
thus offer new, clinically relevant perspectives to attenuate
innate immunity in the context of tolerance induction.

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2.2 Paper II - Dextran sulfate Modulates MAP Kinase Signaling to Reduce Ischemia/reperfusion Injury in a Rat Aortic Clamping Model

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Mitogen-activated protein kinases (MAPKs), including ERK1/2, JNK, and p38, are important players in ischemia/reperfusion (I/R) injury. We recently described that low molecular weight dextran sulfate (DXS, MW 5000), an inhibitor of complement and coagulation, is able to reduce acute myocardial I/R injury. In the present study we hypothesized that targeted cytoprotection by DXS might modulate intravascular inflammation by influencing complement and MAPKs activation. In conclusion, local application of DXS into ischemic vasculature immediately prior to reperfusion reduces complement deposition, and preserves endothelial integrity, partially through modulating activation of MAPKs and may offer a new approach to tackle I/R injury.

Dextran sulfate reduces activation of MAPK and complement deposition. Ischemia/reperfusion injury leads to activation of all three pathways of MAPK as well as to deposition of complement. Local application of DXS into ischemic vasculature reduces complement deposition, downregulates activation of ERK1/2 and p38 MAPK and preserves endothelial integrity.
Dextran sulfate modulates MAP kinase signaling to reduce ischemia/reperfusion injury in a rat aortic clamping model

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Abstract

Mitogen-activated protein kinases (MAPKs), including JNK, p38 and ERK1/2, noticeably influence ischemia/reperfusion (IRI). The complement inhibitor dextran sulfate (DXS) associates with damaged endothelium denuded of its heparan sulfate proteoglycan (HSPG) layer. Other glycosaminoglycan analogs are known to influence MAPK signalling. Hypothetically therefore, targeted intravascular cytoprotection by DXS may function in part through influencing MAPK activation to reduce IRI-induced damage of the vasculature.

IRI of the infrarenal aorta of male Wistar rats was induced by 90 min clamping followed by 120 min reperfusion. DXS (5 mg/ml) or physiological saline (NaCl controls) was infused locally into the ischemic aortic segment immediately prior to reperfusion. 90 min ischemia-only and heparinase infusion (maximal damage) experiments, as well as native rat aorta, served as controls. Aortas were excised following termination of the experiments for further analysis.

DXS significantly inhibited IRI-induced JNK and ERK1/2 activation (p=0.043; p=0.005) without influencing the p38 pathway. Reduced aortic injury correlated with decreased nuclear factor kappa B translocation within the aortic wall. DXS treatment clearly reduced complement deposition, whilst preserving EC integrity and reducing reperfusion-induced HSPG shedding. Protection was associated with specific binding of fluorescein-labeled DXS to ischemically damaged tissue.

Local application of DXS into ischemic vasculature immediately prior to reperfusion reduces complement deposition, and preserves endothelial integrity, partially through modulating activation of MAPKs and may offer a new approach to tackle IRI in vascular surgical procedures.
Introduction

Mitogen-activated protein kinases (MAPK), comprising extracellular signal-regulated kinases (ERK1/2), c-Jun NH2-terminal kinase (JNK) and p38-MAPK, are a multigene family of serine / threonine protein kinases, which respond to a wide variety of stimuli, including environmental stresses and growth factors (1).

Several studies have demonstrated a role for the MAPK family in ischemia/reperfusion injury (IRI). Activation of stress-induced JNK as well as p38 MAPK occurs upon ischemia and ensuing reperfusion (2, 3), significantly contributing to target organ injury. Inhibition of the JNK and p38 MAPK pathways protects ischemic and reperfused heart (4, 5). Conversely the ERK1/2 pathway has been shown to be protective (6). However, the situation is complex; a dynamic balance of the activities of these kinases may prove critical in deciding the outcome post IRI. Endothelial cell (EC) activation is central to IRI. Disruption of the surface-protective endothelial glycocalyx layer (7), upregulation of endothelial adhesion molecules (8) as well as loss of anti-coagulant properties (9) promotes a pro-inflammatory and pro-coagulant environment. Furthermore, activation and local upregulation of complement proteins (10) plays a pivotal role in mediating tissue damage.

Complement-mediated induction of MAPK pathways has been described, including ERK1/2 and JNK activation, in the induction of mitotic signaling in a variety of cells (11) as well as chemotaxis in neutrophils through C5a receptor signaling(12).

Low molecular weight dextran sulfate (DXS, MW 5000), a glycosaminoglycan analog, is a well-characterized inhibitor of complement and coagulation pathways (13-15) and associates with EC denudated of their glycocalyx heparan sulfate proteoglycans (HSPG) (16).

We have observed, that DXS not only localizes to the cell surface, but is also found intracellularly and inhibits phosphorylation of IkappaB-alpha and activation of nuclear factor kappaB (17). DXS may therefore influence intracellular signaling pathways. We postulated that, like other glycosaminoglycans (18), DXS might also modulate MAPK signaling. The aim of this study therefore was to investigate aortic IRI in a rat model and whether association of DXS with damaged endothelium and its known cytoprotective and complement-inhibitory effects correlate with a modulation of the MAPK pathways. This may be of particular interest in vascular surgery, where numerous vessels, including the aorta, may be subjected to longer periods of ischemic assault during, amongst others, complex surgical (re-vascularisation) procedures.
Materials and methods

Materials

Low molecular weight DXS (sodium salt, MW 5000) was purchased from Sigma Chemical Company (St Louis, MO, USA). Fluorescein-labeled DXS (DXS-Fluo) was produced using fluorescein cadaverine (Molecular Probes Europe, Leiden, The Netherlands) as described earlier (16). It was verified that DXS-Fluo essentially had identical biochemical properties as the unlabeled substance.

Experimental procedure

Care and use of animals in the present study was in compliance with national as well as international guidelines.

A total of 80 male Wistar rats (270±65 g) were anesthetized by isoflurane / oxygen, through a semi-closed circuit inhalation system. Following a midline abdominal incision, the infra-renal aorta was mobilized as described by Neil et al. (19). After preparation, the aorta was clamped just distally of the renal arteries and carefully flushed with 1 ml of physiological saline to remove any traces of blood. A second clamp was placed approximately 1 cm distally of the first clamp, proximally of the aortic bifurcation. After 90 minutes of ischemia, 200 µl physiological saline (NaCl, controls, n=20) or 200 µl of 5 mg/ml or DXS in NaCl (n=20) were infused into the lumen of the ischemic aortic segment. Five minutes later, first the distal, then the proximal clamp were released and the aortic segment was reperfused for 120 minutes.

Fifteen ischemia-only control experiments were performed in which the animals were sacrificed and the aortas excised after 90 minutes of clamping without reperfusion. In a further 15 experiments, DXS-Fluo was used instead of the normal, unlabeled substances. These experiments were done in order to evaluate the binding of DXS to the endothelium and aortic wall.

At the end of the experiments, the rats were sacrificed and the aortas were harvested for further evaluation.

Heparinase III treatment

With the hypothesis that endothelial HSPG release may play an important role in IRI and that this release prompts DXS binding, rats were subjected to aortic clamping for 10 minutes, during which 100 µl heparinase III (1 U/ml, Heparinase III from
*Flavobacterium heparanum* (E.C.4.2.2.8), an enzyme selective for heparan sulfate within the glycocalyx, Fluka Chemie, Buchs, Switzerland) was injected into the aortic lumen (n=6). Controls included 10 minutes incubation with heparinase III-buffer only (n=4). Following incubation with heparinase III or buffer, the aortas were either excised immediately or reperfusion was induced. In further experiments, 200 µl DXS-Fluo was injected into the aortic segment after heparinase treatment, followed by reperfusion. All rats were kept under general anesthesia throughout the experiments, including the reperfusion phase.

**Immuno-staining**

Rat aortic rings were retrieved (ischemic segment, non-ischemic control segment), rinsed carefully with saline, embedded in Tissue-Tek OCT compound (Sakura Finetek Europe BV, Zoeterwoude, The Netherlands), and stored at -80°C until further analysis. Five µm sections were air-dried, acetone fixed, hydrated and labeled using a two-step indirect immunofluorescence technique or immunohistochemical approach. The following antibodies were used: rabbit antisera specific for human C1q, C3b/c and C4b/c (Dako), rabbit anti-rat C9 (kind gift of Prof. Paul Morgan, Cardiff, UK), monoclonal mouse anti-human HSPG (Seikagaku, Tokyo, Japan), rabbit antiserum specific for human von Willebrand Factor (vWF, Dako), and rabbit anti-human tissue factor (TF, American Diagnostica Inc, Stamford, USA). Cross-reactivity with the respective rat antigens was verified for all used antibodies and antisera.

Secondary antibodies used were goat anti-rabbit IgG(H+L)-FITC (Southern Biotechnology Associated, Birmingham, AL, USA) sheep anti-rabbit(F(ab')2)-Cy3 (Sigma) and rabbit anti-mouse(Ig)-FITC (Dako).

Staining for phospho-JNK and phospho-ERK1/2 was performed with polyclonal antibodies specific for phosphorylated JNK and ERK1/2 respectively (Cell Signaling, Bioconcept, Switzerland) followed by secondary labeled polymer using the Envision HRP Kit and diaminobenzidine chromogen kit (both Dako). After washing, the slides were counterstained with hematoxylin. All slides were mounted with SlowFade Light Antifade Kit (Molecular Probes Europe, Leiden, The Netherlands).

Nuclear factor (NF) kappaB was visualized using a rabbit anti-NF kappaB p65 (Abcam, Cambridge, MA) antibody at 1:50 dilution, followed by secondary antibody (Dako) and counterstaining with hematoxylin.
Images were acquired with a fluorescence microscope (Nikon Eclipse TE2000-U) and digital camera (Nikon DXM1200F). Images were captured with identical exposure times and settings in each experiment. For quantification of fluorescence intensity, Image J software (http://rsb.info.nih.gov/ij/) was used, in addition to grading of four samples per experiment with respect to staining intensity (as a further control to quantify staining): 0 = essentially no staining, 1 = minimal focal or diffuse staining, 2 = moderately strong focal or diffuse staining 3 = extensive diffuse staining. For NF kappaB staining six random fields were chosen and nuclei staining positive for NF kappaB were counted and divided by the total number of nuclei identified in the field.

**Western blotting for MAP kinases**

Aortic segments were snap-frozen in liquid nitrogen and kept at -80°C until further processing for Western Blotting. Cell extracts were prepared by lysing cells from the aortic tissue samples in extraction buffer as described previously (20). Extracts of 40 µg were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrophoretically transferred to an Immobilon-P membrane (Millipore, Billerica, MA, USA). Following blocking with 5 % skimmed milk, the membrane was incubated overnight at 4°C with the corresponding primary antibody (polyclonal antibodies raised in rabbits for detection of JNK, ERK1/2, p38 MAPK and their respective phosphorylated counterparts; all Cell Signaling, Bioconcept, Switzerland). This was followed by washing and incubation with an appropriate anti-rabbit alkaline phosphatase-conjugated secondary antibody. Signal was detected by Western Blue (Promega, Madison, WI, USA) stabilized substrate. Quantification of the signals was performed with NIH Image 1.62 software.

**Statistics**

Differences between as well as within the experimental groups were analyzed by one-way-analysis of variance (ANOVA). Differences were considered to be significant with a p-value of <0.05. Data, unless otherwise specified, are presented as average ± standard deviation in text and figures.
Results

Tissue complement deposition

DXS treatment markedly reduced complement deposition after I/R compared to the NaCl control group: C1q (mean grading score; 1.0 ± 0.7 vs. 2.3 ± 0.7), C4b/c (2.1 ± 0.8 vs. 0.8 ± 0.7), C3b/c (2.3 ± 0.7 vs. 1.0 ± 0.7) and C9 (0.9 ± 0.7 vs. 2.1 ± 0.8) (scoring not shown). This reduction was evident in stained tissue sections (Figure 1), quantitatively represented as histograms following analysis with image J software (Figure 2). Minimal complement deposition on the endothelial surface was detected after ischemia-only (mean grading score: C1q 0.4 ± 0.5; C4c 0.4 ± 0.5; C3c 0.8 ± 0.7; C9 0.3 ± 0.5, not shown). A similar, though more extensive staining pattern of complement deposition was observed after 10 minutes heparinase treatment followed by reperfusion as compared to the NaCl ischemia / reperfusion control group. No complement deposition was detected upon heparinase administration without reperfusion or heparinase buffer only (not shown).

![Figure 1. Immunofluorescence staining for complement C1q, C4b/c, C3b/c and C9 on rat aortic tissue.](image)

Essentially no or minimal complement staining following ischemia-only (I-only). Complement activation and deposition after 90 min ischemia / 120 min reperfusion following NaCl administration as well as after infusion of heparinase (Hep III) / 120 min reperfusion. Dextran sulfate (DXS) clearly reduces complement deposition. Scale bare represents 100µm. All images are representative of at least 4 scored sections per experiment.
Tissue factor expression

As compared to native controls (normal), tissue factor expression markedly increased in the NaCl control group at the end of the 120-minute reperfusion phase as compared to aortas from DXS-treated animals and ischemia-only controls (Figure 4a). Image J software quantification of fluorescence intensity (Figure 4b).

![Fluorescence intensity plots for complement components C1q, C3c/b, C4c/b, and C9](image)

**Figure 2.** Quantification of fluorescence intensity for complement staining / deposition of C1q, C4b/c, C3b/c and C9. Histograms represent an average of staining intensity (fluorescence, x-axis) from images from at least 4 individual experiments. Shift of the curve towards the right indicates increased intensity rsp. increased complement deposition.

Binding of fluorescein-labeled dextran sulfate (DXS-Fluo)

DXS-Fluo binding was only detected in aortas subjected to ischemia / reperfusion or heparinase treatment and was not found on native aorta or after heparinase buffer alone (Figure 5). No DXS-Fluo binding was identified in other inspected organs (lungs, liver, kidney, spleen; not shown).
p38 MAPK: Western blot

p38 MAPK phosphorylation was significantly increased in the NaCl controls (p=0.022, Figure 6a) and heparinase III treated animals (p=0.007, not shown) as compared to normal aorta. Neither ischemia-only nor DXS treatment significantly affected p38 activation (DXS: p=0.110; ischemia only: p=0.440 vs. normal).

Figure 3. Von Willebrand (vWF) and heparan sulfate proteoglycan (HSPG) staining on rat aorta. Circumferential positive staining for vWF in all experimental settings. Circumferential surface and wall HSPG staining in ischemia-only (I-only) controls and DXS treated aorta. Diminished / abrogated HSPG staining in NaCl controls and heparinase-treated aortas. Scale bare represents 100µm. All images are representative of at least 4 scored sections per experiment.

ERK1/2: Western blot and staining

ERK1/2 phosphorylation was significantly increased in all experimental groups as compared to normal aorta (p<0.05 for all groups, Figure 6b). ERK1/2
phosphorylation was not significantly higher in NaCl controls as compared to ischemia-only experiments (p=0.064). DXS treatment significantly reduced

**Figure 4. Immunofluorescence staining.** (A) for tissue factor. Yellow arrowheads highlight surface tissue factor expression. Marked increase in tissue factor expression in the NaCl control group as compared to aortas from DXS-treated animals and ischemia-only controls (I-only). Native (normal) aortas are used as a control. All images are representative of at least 4 scored sections per experiment. Image J software quantification of fluorescence intensity (B). Histograms represent an average of staining intensity (fluorescence, x-axis) from images from at least 4 individual experiments. Shift of the curve towards the right indicates increased intensity r.s.p. increased complement deposition.

ERK1/2 activation as compared to NaCl controls (p=0.005). Immunohistochemical staining for activated ERK1/2, using an antibody specific for phosphorylated ERK1/2, confirmed increased levels in the NaCl controls as compared to the DXS treated animals.
Figure 5. **Binding of dextran sulfate.** Specific binding of fluorescein-labeled DXS (DXS-Fluo) to ischemically damaged aorta as well as heparinase-treated aorta. No binding found to non-ischemic aorta. Scale bar represents 50µm.

**JNK: Western blot and staining**

c-Jun NH2-terminal kinase (JNK) phosphorylation was significantly increased in all experimental groups as compared to normal aorta (p<0.005 for all groups, Figure 6c). DXS administration prior to reperfusion significantly inhibited JNK phosphorylation as compared to NaCl controls (p=0.043), down to levels of ischemia-only. Immunohistochemical staining for activated JNK, using an antibody specific for phosphorylated JNK, confirmed homogenously increased levels in the NaCl controls as compared to the DXS treated animals.

**NF kappaB staining**

Immunohistochemistry revealed that the degree of IRI and complement activation correlated with the extent of NF kappaB nuclear translocation in the aortic wall. Minimal nuclear NF kappaB was observed in native aortas (Figure 7). However, there was substantial nuclear NF kappaB localization in the control NaCl treated aortas, with evidently less translocation following DXS administration.

**Discussion**

Ischemia-induced damage, not only to the end organ but also to the vasculature itself is of importance, particularly in vascular surgery that may necessitate temporary vascular clamping. Resulting reperfusion may lead to substantial endothelial damage with HSPG release and denudation, ensuing activation of complement and coagulation, resulting in potentially extensive (intra-)vascular damage as well as end organ injury. Indeed, in ischemia/reperfusion injury (IRI), complement activation (21) and the effectiveness of its inhibition in providing protection to various organs has been demonstrated in a multitude of models (22, 23). Here too, we show that the glycosaminoglycan analog DXS attenuates IRI-induced aortic complement activation and damage.
Figure 6. Western Blot results for MAPK activation. Ratio of phosphorylated (activated) p38 MAPK to p38 MAPK in aortic tissue samples from normal, ischemia-only (I-only), NaCl controls and DXS-treated animals (A). Significant increase in the NaCl group as compared to normal aorta (p=0.02). DXS treatment and ischemia-only did not affect p38 MAPK activation (p=0.110, p=0.440 respectively). Ratio of phosphorylated (activated) c-Jun NH2-terminal kinase (JNK) to JNK in aortic tissue samples (B). Significant increase in all groups as compared to normal aorta (p<0.005). DXS treatment significantly reduced JNK activation versus NaCl controls (p=0.043). Ratio of phosphorylated (activated) ERK1/2 to ERK1/2 in aortic tissue samples (C). Significant increase in all groups as compared to normal aorta (p<0.005). DXS treatment significantly reduced ERK1/2 activation versus NaCl controls (p=0.005). All data are mean ± standard deviation. Representative images from immunohistochemical staining for JNK and ERK1/2 (B and C) reveal reduced staining in native / normal and DXS aortas as compared to control NaCl aortas (brown colour).
Figure 7. Representative images from immuno-histochemical staining for nuclear factor kappa B reveals reduced nuclear staining in native / normal and DXS aortas as compared to control NaCl aortas. Nuclear positivity appears as a brown staining (see arrow heads in inlay). Scale bar represents 50µm.

These results are in line with our own work and previous studies, where the use of glycosaminoglycan analogs such as heparin, reduced IRI, through complement inhibition and endothelial association (24, 25).

Whilst glycosaminoglycans, including heparin as well as cell-surface HSPG have been reported to modulate MAPK signaling (26, 27), complement itself influences MAPK activation. Sublytic amounts of C5b-9 activate the JNK and ERK pathways, inducing mitotic signaling in a B-cell line and smooth muscle cell proliferation in aortas (28). Also, constitutive ERK activation may exacerbate complement-mediated injury to glomerular epithelial cells (29). Inhibition of MAPK activation, for instance the stress-activated JNKs and p38-MAPKs has been shown to reduce injury, also complement-mediated, in various target organs and models (5, 30). To date, however, it remains unknown, whether complement inhibition and accompanying modulation of MAPK signaling may be beneficial in reducing IRI-induced tissue injury. Our results indicate that complement inhibition with DXS is associated with reduced JNK and ERK1/2 activation. This, in itself may provide one way by which DXS modulates the pro-inflammatory events associated with ischemia and reperfusion. The reduction in MAPK activation is accompanied by reduced tissue factor expression in the DXS treated aortas and correlated with reduced NF kappaB.
nuclear translocation, as we have already previously reported in an in vitro setting (17). Influencing signaling pathways through modulation of protein kinases may prove an effective strategy to tackle a variety of diseases (31). However, there remains a degree of uncertainty as to whether inhibition or indeed activation of MAPK, proves beneficial or detrimental (32). In particular, effects may also be species- and model-dependent (33).

Binding of DXS to the damaged endothelium and aortic wall, visualized using fluorescein-labeled DXS was observed following local administration. Binding was specific for ischemically damaged tissue and was not detected in non-ischemic aorta or other organs. DXS was also detected on the endothelium and within the aortic wall following pre-treatment of the aorta with heparinase III, which, like other enzymes (34) removes HSPG from the endothelial surface. This finding correlates with previous work, demonstrating increased binding of DXS to porcine endothelial cells following heparinase treatment (16).

The pharmacologically-induced HSPG release mimicked the HSPG loss detectable after 90 minutes ischemia, which was amplified following subsequent reperfusion. DXS, however, largely prevented reperfusion-induced HSPG shedding. However, the increased staining intensity after DXS administration (Fig. 3) may at least partly be due to cross-reaction of the anti-heparan sulfate antibody with endothelial-bound DXS. Crossreactivity of this antibody with DXS was recently confirmed in a competitive inhibition experiment (35).

Heparan sulfate, like medicinal heparin, enhances the activity of antithrombin, thereby contributing to the inactivation of thrombin and activated FXa, respectively (36). Heparinase treatment of endothelial cells reduces antithrombin binding, suggesting that this interaction is indeed mediated by heparan sulfate (37). Replacing the shed HSPG layer by a glycosaminoglycan analog, such as DXS, or indeed, preventing reperfusion-induced shedding, may help restore this essential property of “surface regulation” of the coagulation system.

In conclusion, we provide evidence that low molecular weight DXS substantially reduces tissue damage following ischemia and reperfusion in an infrarenal aortic clamping model in rats. Although DXS may primarily provide cytoprotection through association with denudated endothelium and local complement inhibition, it remains to be further clarified what role the modulation of the MAPK pathway plays in this setting. Local cytoprotection of damaged endothelium, using substances such as DXS, which may functionally restore the anti-inflammatory and anti-coagulant properties of the luminal glycocalyx, may provide a novel way to attenuate vascular, as well as end organ reperfusion-induced injury in the clinical setting, for example in vascular surgery.
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References


2.3 Paper III – Analytical Aspects and Limitations of Mass Spectrometry-based Methods in Serum Hepcidin Quantification

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The hepatic peptide hormone hepcidin is a key regulator of iron homeostasis. Quantification of serum hepcidin in patients would therefore help to understand its role in the development of anemia, for example, as a consequence of cardiovascular surgery. To date, there is no reliable method available for the determination of hepcidin in serum. We investigated the possibility of establishing a method for quantitation of human serum hepcidin based on liquid chromatography/tandem mass spectrometry (LC-MS/MS) and reached the conclusion that the reliability of LC-MS/MS-based method may not the standard for clinical quantification of serum hepcidin as a result of strong oxidation. Non-specific binding might be an additional factor.

Role of hepcidin in iron homeostasis. Most of the utilized body iron is recycled from senescent erythrocytes by macrophages and returned to the bone marrow for incorporation in erythroid precursors. The liver and reticuloendothelial macrophages function as major iron stores. Hepcidin controls the plasma iron concentration by inhibiting iron export by ferroportin from enterocytes and macrophages. This means that an increased hepcidin production, a circumstance like inflammation associated with surgery/transplantation, leads to a decrease in plasma iron concentrations.
Analytical aspects and limitations of mass spectrometry-based methods in serum hepcidin quantification

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Abstract

The hepatic peptide hormone hepcidin is a key regulator of iron homeostasis. Quantification of serum hepcidin in patients would therefore help to understand its role in the development of anemia, for example, as a consequence of cardiovascular surgery. To date, there is no reliable method available for the determination of hepcidin in serum. We investigated the possibility of establishing a method for quantification of human serum hepcidin based on liquid chromatography/tandem mass spectrometry (LC-MS/MS).

The method consists of serum protein precipitation and solid phase extraction. Then the samples were subjected to LC-MS/MS analysis. In a series of experiments, synthetic hepcidin standard was spiked into rabbit serum as a surrogate matrix and the recovery was evaluated with the internal control.

Pure hepcidin standard could be detected at amounts as low as 25 fmol/µl. The integrated extracted ion peak area of the three detectable charge state ions of hepcidin correlated well with the injected amount of peptide. The presence of the three parent ions and the three collision-induced dissociation (CID) daughter ions at the same retention time gives this assay a very high specificity. However, spiking of hepcidin standard into rabbit serum revealed that the recovery of the peptide was random and correlated poorly with the spiked amounts. This might be due to non-specific binding of hepcidin to the surfaces, and oxidation during sample preparation steps might limit the quantification in LC-MS/MS based methods. In conclusion, the reliability of LC-MS/MS-based method is currently not sufficient for clinical quantification of serum hepcidin.
Introduction

A novel highly disulfide-bonded human peptide was isolated in the year 2000 from a plasma ultrafiltrate and named liver-expressed antimicrobial peptide-1 (LEAP-1), which shows antimicrobial activity in vitro (1). In the same year, it was also isolated from human urine and named hepcidin (hepatic bactericidal protein), based on its origin in the hepatic tissue and in vitro bactericidal activity (2). Hepcidin gene knock out in mice (Usf2 -/- mice) demonstrated severe iron overload and suggested that hepcidin plays an important role in iron metabolism (3). Conversely, transgenic mice expressing hepcidin constitutively die at birth with severe iron deficiency (4).

The human hepcidin molecule consists of 25 amino acids and negatively regulates iron uptake from the intestine (5), release from the hepatic store and recycling by macrophages (6), which are the three main gateways of maintaining the body iron homeostasis. Hepcidin synthesis is greatly increased within one day after an iron rich diet in mice and in humans (7). However, the hepcidin-mediated mechanism of iron regulation remains elusive. Iron absorption from the intestine and release from the body iron store are greatly enhanced by erythropoietic stimuli associated with blood loss and anemia, whereas hepcidin gene expression is markedly down regulated in blood loss and hypoxia (8), which may account for the increased iron absorption and iron release from the reticulo endothelial system. Thus, the serum hepcidin level is markedly reduced during erythropoietic stimuli. The serum hepcidin level is markedly increased during infection independent of blood loss or hypoxia (9). It has been shown that IL-6 is necessary and sufficient for the upregulation of hepcidin in inflammation (7). Anemia of inflammation is associated with chronic and acute infection, generalized inflammatory disorders and malignancy. Cytokines, particularly IL-6, induced during inflammation upregulate hepcidin. Subsequently, iron supply is limited in bone marrow for erythropoiesis.

Currently, quantitative detection of hepcidin is a challenging task for understanding the clinical pathogenesis of hepcidin-related ion homeostasis. Hepcidin mRNA analysis is preferred in animal models and in vitro cell culture systems. Due to the invasiveness of sampling, it is not a suitable method in human studies. The other methods such as immunohistochemical staining, western blot and ELISA methods are also limited because of the lack of reliable anti-hepcidin antibodies. Although western blot-based serum hepcidin data have been published, the diagnostic value is poor due to a lack of specificity of the antibody used in the method (10). The difficulty in producing a suitable antibody is attributed to the small size of the hepcidin molecule (2.7 kDa), its compact structure due to the high number of disulfide bonds and its highly conserved sequence in species, which prevents boosting immune
activation upon antigen challenge. Most of the widely used antibody-based ELISA is designed to analyze serum prohepcidin, which consists of 60 amino acids with a molecular mass of 10 kDa (11). Measurement of prohepcidin has a poor correlation with other ion related parameters. However, very recently Ganz et al. described an immunoassay for hepcidin (12).

Non-antibody based methods, such as mass spectrometer related analysis strategies, have also been described. A surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS)-based assay has been reported for quantification of serum hepcidin (13, 14). Recently, liquid chromatography/tandem mass spectrometry (LC-MS/MS) based quantitative methods for the determination of both human and mouse serum hepcidin in serum and plasma have also been proposed (15). However, the use of mass spectrometer based methods has not been reported to quantify serum hepcidin in a clinical setting. Hence, here we describe the analytical aspects and limitations of the LC-MS/MS-based analytical method to quantify serum hepcidin.

**Materials and Methods**

**Spiked assay**

Hepcidin standard was purchased from Bachem, Bubendorf, Switzerland and reconstituted in 0.1% trifluoroacetic acid in water. As described previously, rabbit serum was used as a surrogate matrix (15). Rabbit serum was spiked with 25, 5, 1.5 and 0.5 ng of hepcidin.

**Internal standard**

Adding an internal standard enables the control of the instrumental settings and evaluation of losses during sample preparation. Internal standards were included at two different points of the procedure. After spiking the rabbit serum with hepcidin, 5 ng of calcitonin gene related peptide (CGRP, Sigma, Buchs, Switzerland) was added to the all samples. Bombesin at a final concentration of 200 fmol/µl was added as additional control before LC-MS/MS.

**Extraction of hepcidin**

300 µl of acetonitrile (Merck, gradient HPLC grade) were added to 100 µl of rabbit serum spiked with hepcidin and CGRP. The mixture was vortexed for 20 sec, left to stand for 5 min and centrifuged at 4 °C for 10 min at 4000 rpm. After centrifugation, 200 µl of supernatant was transferred to a Waters Oasis HLB extraction cartridge.
(1 ml/10 mg) containing 800 µl of distilled water. The HLB extraction cartridge was washed with water and then 5% acetonitrile. Elution was performed by adding 1 ml of 0.05/20/80 TFA/water/ACN (v/v/v). Each fraction was added with 25 µl of 1% NaCl (250 µg) and 1 mM DTT (Sigma, Buchs, Switzerland). Samples were evaporated to reduce volume at 50 °C using an evaporator (Zymark). Samples were further dried in a speed vac system at medium heat for approximately 45 min.

**Liquid chromatography/Mass spectrometry**

Each residue was reconstituted in 25 µl of 20% formic acid (with or without 200 fmol bombesin peptide) by incubation at room temperature with occasional vortexing. Samples were centrifuged at 4°C for 10 min at 15400 rcf, followed by transfer of 20 µl supernatant to HPLC vials. Then 15 µl was loaded on the column and analyzed by LC-MS. Separation of the sample on a 50 x 0.15 mm RP column (GROM-SIL MB6 C8, 5 µm, 300Å) with a gradient of 2-5% MeCN in 1 min, 5-40% MeCN in 20 min, 40-80% MeCN in 5 min at a flow rate of ca. 3 µl/min. Solvents contained water and 0.1% (v/v) formic acid. MS/MS acquisition was done on an Esquire3000plus iontrap (Bruker) and on an Orbitrap (ThermoFisher) with targeted CID on the hepcidin and CGRP ions. For quantification purposes, the EIC peak areas of the three major ion species of each polypeptide were used.
Results

Quantification of pure hepcidin using LC-MS/MS

Using the LC-MS/MS method, pure hepcidin can be reproducibly detected even at a load of 25 fmol (Figure 1). The integrated extracted ion peak area of the three detectable charge state ions of hepcidin correlates with the injected amount of peptide. The summed peak areas of the three most prominent daughter ions with \( m/z > 1000 \) in the collision-induced dissociation (CID) spectrum of the 3+ parent ion of \( m/z 930.5 \) also correlate with the amount of hepcidin loaded onto the columns. The presence of the three parent ions and the three CID daughter ions at the same Rt gives this assay a high specificity.

![Figure 1. Quantification of pure hepcidin. Hepcidin at the concentration of 1 mg/ml was diluted 1:349 in 20% (v/v) formic acid (approximately 1 pmol/\( \mu l \)) and 4 \( \mu l \) of this pre-dilution were further diluted in 156 \( \mu l \) of a peptide matrix, consisting of 1 \( \mu g \) of a digest of twelve standard proteins in 20% (v/v) formic acid resulting in a hepcidin end concentration of 25 fmol/\( \mu l \). Volumes of 1, 2, 4, 6, 8, 10, and 20 \( \mu l \) of this solution, and 1 \( \mu l \) of the 1 pmol/\( \mu l \) solution was injected twice each (25, 50, 100, 150, 200, 250, 500, 1000 fmol). Data dependent LC-MS/MS acquisitions were made with MS set to isolate only 3+ ion of hepcidin (m/z = 930.5) for CID. The extracted ion chromatographic peaks (EIC) of parent (3+, 4+, and 5+ ions with m/z of 930.5, 698.2, and 558.8, respectively) and daughter ions of m/z 1086, 1145.5, and 1218.5 were integrated for quantification (sum of daughter iron areas is shown).
Figure 2. Ion chromatograms of hepcidin in rabbit serum. Approximately 500 fmol hepcidin was spiked into 100 ul of rabbit serum and processed as described in materials and methods. Panel A is the total ion chromatogram. In panels B, C, and D the extracted ion chromatograms of hepcidin, oxidized hepcidin and CGRP are shown. Ion intensities were extracted from the four most intense isotopic peaks from the +4, +3, and +2 hepcidin ion species (monoisotopic m/z of 697.7637/701.7624, 930.0159/935.3475, 1394.5202/1402.5176) and +5, +4, +3 CGRP ion species (m/z = 758.3953, 947.7422, 1263.3216), respectively. Panel E is the extracted ion intensity peak for bombesin with m/z values of 810.4115, 810.9127, and 811.4133. The apex peak height is given on top of each peak maximum. Note that the pure peptide standards with 500 fmol loaded onto the column resulted in peak heights of 17'467'646 for hepcidin, 5'915'980 for CGRP, and 24'081'056 for bombesin, while oxidized hepcidin was barely detectable.
Iron chromatograms of hepcidin in rabbit serum

Pure hepcidin standard at approximately 500 fmol was spiked into rabbit serum with CGRP. Analysis of the iron chromatogram revealed that hepcidin, oxidized hepcidin and CGRP were readily detectable (Figure 2).

Figure 3. Distribution of the charge state of hepcidin. The charge state distribution of hepcidin standard analyzed by LC-MS on the Orbitrap is shown in panel A. The peaks of charge states +4, +3, and +2 were used for ion peak extractions. The +3 ion isotopes shown in panel B belong to the native, unmodified hepcidin. Hepcidin becomes readily oxidized as shown in panels C and D, with the form shown in D being additionally two times deamidated.

The charge state distribution of hepcidin standard

The charge state distribution of hepcidin standard analysis revealed a considerable amount of the oxidized form of hepcidin in addition to hepcidin (Figure 3). This may be due to the fact that during the sample preparation, hepcidin is subjected to oxidation and subsequently detected in LC-MS/MS.

Hepcidin spiked with the internal standard

Analysis of hepcidin standards responds almost linearly in LC-MS/MS (Figure 4). However, 2% of the hepcidin standards were oxidized. The CGRP response in a theoretically equimolar concentration as hepcidin is about 30% compared to hepcidin standards. Bombesin was used as an internal standard and was spiked after the solid phase extraction. Spiking bombesin after the SPE provides accurate estimation of the yield of hepcidin after SPE. The bombesin response was identical to hepcidin
assuming equimolar concentrations of hepcidin and Bombesin.

Figure 4. Spiking hepcidin with the internal standard. Hepcidin & CGRP (1 mg/ml) were diluted 1:36 and 1:26, respectively, into a diluted aliquot of a human umbilical vein endothelial cell (HUVEC) lysate, digested in 20% formic acid. Then, 2 µl of this dilution was further diluted in 96 µl of 20% formic acid and 2 µl of a peptide standard. The resulting theoretical concentration of each peptide has 0.2, 2.5, 5 pmol/µl, and 10 µl of each standard peptide dilution was loaded and analyzed by LC-MS.

Correlation of spiked hepcidin and the internal standards

Spiking of hepcidin standard into rabbit serum revealed that the recovery of the peptide was random and correlated poorly with the spiked amounts (Figure 5). Oxidized hepcidin was found in almost all samples and the levels of oxidized hepcidin varied widely from sample to sample. Recoveries of hepcidin and CGRP from the four spiked serum samples appear almost random and do not correlate with the theoretically increasing hepcidin concentration. However, the measured CGRP and hepcidin signals do correlate somewhat. The higher the bombesin signal, the more intense was the measured hepcidin signal. This correlation does not apply for bombesin vs. CGRP. Based on the bombesin signal, there were between 0.01 pmol (1.5 ng = 0.5 pmol) and 0.27 pmol (0.5 ng = 0.15 pmol) hepcidin recovered in the eluates.
Discussion

Although several studies on mass spectrometry-based analysis of hepcidin have been published, use of these methods for quantitative detection of hepcidin in serum is not yet practiced in clinical settings. In this study we investigated analytical aspects and limitations of the mass spectrometry based methods of quantitative detection of hepcidin. It is possible to find in circulation that there are both carrier bound and free forms of hepcidin (10).

![Figure 5. Hepcidin spiked assay.](image)

Samples were dried in a speed vac system at the beginning with medium heat, then heating was switched off before samples were dry. Residues were reconstituted in 25 µl of 20% formic acid containing approximately 40 fmol/µl of bombesin for 20 min at room temperature with occasional vortexing. The samples were centrifuged at 4°C for 10 min at 15'400 RCF. Then 22 µl of supernatant were transferred to HPLC vials and 18 µl were analyzed by LC-MS with targeted MS/MS on used isotopes of bombesin, hepcidin (4+/3+ ions) and CGRP (5+ ion).

Use of a high ratio of acetonitrile for protein precipitation has been shown to provide the best protein precipitation efficiency (16). Hence, the used serum-acetonitrile ratio of 1:3--would be ideal for serum protein precipitation, which is useful for LC-MS/MS analysis. However, hepcidin may form a complex with a higher molecular weight in serum (10). Therefore precipitation of the serum protein using acetonitrile might also
precipitate a substantial amount of hepcidin due to the high molecular weight of the carrier proteins.

It has been considered that hepcidin has a strong non-specific binding capacity to the surfaces during sample preparation and this appears to result in fewer recoveries in LC-MS/MS based methods (17). Increased recovery of hepcidin is likely associated with decreased sticking of hepcidin with the increase of the volume to surface ratio or to less aggregation with the decrease of the hepcidin concentration (13). Increasing the volume in our experiments up to 1000 µl in the collecting tubes or use of minimum concentrations did not enhance the recoveries evaluated in LC-MS/MS.

Oxidation of hepcidin also appears to limit the quantification in LC-MS/MS based methods. Oxidation of hepcidin in urine hepcidin samples has also been reported. In urinary samples the methionine residues of hepcidin 24 (2673.9 Da) and hepcidin 25 (2789.4 Da) were subjected to oxidation at experimental conditions (13, 18). This ambient ozone-induced oxidation artifact was minimized by carrying out sample preparation in a nitrogen atmosphere using an incubator with a nitrogen inlet (13). Oxidation peaks of hepcidin in the MS spectra were not detected in urine samples prepared under a nitrogen atmosphere, whereas they were clearly visible in some spectra of samples loaded on the arrays in ambient air (13). Sample handling in a nitrogen atmosphere would be not feasible in routine diagnostic conditions. Handling in such conditions would require a special laboratory setup. Although our approach of adding DTT from the very beginning of the experiments appears to be a good strategy, it did not completely prevent oxidation of hepcidin. Therefore, oxidation during sample preparation appears to be the major barrier limiting quantification of LC-MS/MS based methods.

In conclusion, we found that sample preparation methods associated with LC-MS/MS-based analysis might limit the accurate quantitative analysis of serum hepcidin. Hence, our finding urges the establishment of alternative sample preparation methods in spectrometer based analysis of hepcidin in serum. Alternatively, improvement of other approaches, particularly development of antibody based ELISA methods, would be highly desirable.

References


2.4 Paper IV - Low Molecular Weight Dextran Sulfate as Complement Inhibitor and Cytoprotectant in Solid Organ and Islet Transplantation (Review)

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Over the past years, growing evidence has emphasized the importance of the complement system in transplantation and of the need for strategies to regulate its activation. This review paper discusses the influence of low molecular weight dextran sulfate (DXS, MW 5000) on the regulation of complement activation as well as the maturation of dendritic cells (DC) in solid organ and islet transplantation.

Interplay between dextran sulfate, endothelium, dendritic cells and complement. 1) The cell surface of a healthy endothelium is covered by a layer of HSPG. 2) I/R injury leads to activation of the complement system. 3) Activation of the EC leads to shedding of HSPG and their heparan sulfate chains (HS). HS and other molecules act as damage-associated molecular patterns (DAMPs), which are recognized by pattern recognition receptors (PRR). 4) DAMPs are recognized by DC via PRR, which leads to maturation of the cells. DXS has been shown to prevent both complement mediated EC damage and maturation of human DC.
Review

Low molecular weight dextran sulfate as complement inhibitor and cytoprotectant in solid organ and islet transplantation

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ABSTRACT

Complement is an essential part of the innate immune system and plays a crucial role in organ and islet transplantation. Its activation, triggered for example by ischemia/reperfusion (IR), significantly influences graft survival, and blocking of complement by inhibitors has been shown to attenuate IR injury. Another player of innate immunity are the dendritic cells (DC), which form an important link between innate and adaptive immunity. DC are relevant in the induction of an immune response as well as in the maintenance of tolerance. Modulation or inhibition of both components, complement and DC, may be crucial to improve the clinical outcome of solid organ as well as islet transplantation. Low molecular weight dextran sulfate (DXS), a well-known complement inhibitor, has been shown to prevent complement-mediated damage of the donor graft endothelium and is thus acting as an endothelial protectant. In this review we will discuss the evidence for this cytoprotective effect of DXS and also highlight recent data which show that DXS inhibits the maturation of human DC. Taken together the available data suggest that DXS may be a useful reagent to prevent the activation of innate immunity, both in solid organ and islet transplantation.

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1. Introduction

Over the past years, growing evidence has emphasized the importance of the complement system in transplantation and of the need for strategies to regulate its activation. This paper will discuss the influence of low molecular weight dextran sulfate (DXS, MW 5000) on the regulation of complement activation as well as the maturation of dendritic cells (DC) in solid organ and islet transplantation.

1.1. Complement in allograft rejection

That complement is involved in allograft rejection has been known since the seminal studies on graft-specific antibody responses by Terasaki et al. (1964) almost 50 years ago. Still today, the presence of preformed, complement-activating antibodies in the circulation of the recipient, like in ABO-incompatibility and HLA-presensitization, represents a formidable barrier to successful transplantation. The development of therapeutic options to allow for ABO-incompatible transplantation began already 25 years ago (Alexandre et al., 1985; Bensinger et al., 1992) and today ABO-incompatible living-related kidney transplantation is increasingly being performed using specific anti-A/B immunosuppression or plasmapheresis and anti-CD20 antibody treatment (Tyden et al., 2003). However, while we learned how to deal with anti-A/B blood group antibodies, no recipe has been found as yet to reliably prevent or treat rejection due to anti-HLA antibodies, and both acute and long-term effects of the presence of such antibodies in the recipient's circulation are feared (Gicir et al., 2008). Complement inhibitors have been widely applied to treat antibody-mediated rejection in animal transplantation models, notably in xenotransplantation, but with the exception of intravenous immunoglobulins (IVIg) and Cl-inhibitor (Mohacsil et al., 2001; Montgomery et al., 2000; Peraldi et al., 1996; Struber et al., 1999; Tyan et al., 1994) they are currently not used in clinical transplantation.

In addition to activation by graft-specific antibodies circulating in the blood of the recipient, the deposition of complement on graft cells is also triggered by ischemia/reperfusion (IR) injury. In clinical transplantation, brain death of the organ donor, the procurement procedure, and cold ischemia during preservation and transportation of the graft inevitably lead to IR injury, which critically influences allograft survival. Indeed, approaches to inhibit complement activation in IR injury showed beneficial effects in experimental models of transplantation and beyond. The use of soluble complement receptor 1 (sCR1) was shown to attenuate post-ischemic myocardial inflammation and necrosis (Weisman
et al., 1990) and attenuate I/R injury in experimental models of lung and liver transplantation (Lehmann et al., 1996; Schmidt et al., 1998; Stammberger et al., 2000). These findings have led to studies on the mechanism by which the complement system is activated in I/R injury. Taken together, these studies showed that all three major activation pathways of complement are involved in I/R injury, in a rabbit model of myocardial I/R injury participation of the classical complement pathway was demonstrated by use of a specific C1s inhibitor (Buerer et al., 2001). Involvement of the alternative pathway was observed in a mouse model of renal I/R injury in which mice deficient of factor B had less deposition of C3 (Thurman et al., 2003), and recently also an important contribution of the lectin pathway to I/R injury was reported (Moller-Kristensen et al., 2003; Stahl et al., 2003; Thurman et al., 2003). While the precise mechanisms of I/R injury are not yet fully understood it has been shown that neopterin is exposed on endothelial cells (EC) upon ischemia and recognition upon reperfusion by pattern recognition molecules such as mannose binding lectin (MBL), either directly or via binding of naturally occurring IgM, triggering complement activation and subsequent graft damage (de Vries et al., 2004; McMullen et al., 2006; Zhang et al., 2006b).

1.2. Strategies to inhibit complement activation in transplantation and I/R injury

Therapeutic options for complement inhibition in transplantation are possible both on the side of the donor organ and the recipient, and inhibition of complement in the fluid phase and on cell surfaces can be distinguished. Systemic complement depletion can be achieved by the use of coxib venus factor (CVF). Indeed, CVF therapy was successfully used in pig-to-primate transplantation in combination with immunosuppressive drugs (Kobayashi et al., 1997; Leverth et al., 1994), but its effect on I/R injury is less clear: while a reduction of myocardial infarct size was described already 30 years ago (Manko et al., 1978), reports on its effect on cerebral I/R injury are contradictory (Lew et al., 1996; Vashist et al., 1998). Treatment of the recipient with icatibant has been shown to prevent I/R injury and early graft failure in isogenic (Naka et al., 1997) as well as allogeneic lung transplantation (Pier et al., 1998; Stammberger et al., 2000). Similar results were achieved with the use of C1 inhibitor, which was described to reduce early graft damage in a lung transplantation model in the dog (Salvatierra et al., 1997). The beneficial effect of C1 inhibitor was also seen in patients who suffered from acute graft injury after lung transplantation (Struber et al., 1999). C1 inhibitor also binds to graft EC, while still maintaining its function as complement inhibitor (Bergamaschi et al., 2001), and could therefore potentially be used as an additive to organ preservation solutions in order to protect the graft from I/R injury. Attempts in the same direction, namely the development of a membrane-targeted complement inhibitor, were made by Smith and Smith (2001). This construct, termed 'microcoapt', consisting of the first three short consensus repeats of CRII linked to a lipophilic tail to anchor it in cell membranes, was later on successfully used in rat kidney transplantation experiments and in perfusion of human kidneys (Patt et al., 2003).

Another approach to confer protection from complement-mediated damage to the graft is genetic manipulation of the donor organ. Donor pigs expressing human CD59 (DFM), CD95, and/or CD46 (MCP) have been produced for xenotransplantation (McCurt et al., 1995). Research in this direction is being continued, but so far it is technically not feasible to express such genes on a human allograft prior to transplantation. On the other hand, novel fluid-phase complement inhibitors such as Pesetuzumab, an anti-C5 antibody, are being tested in clinical trials in the field of cardiology (Maloof et al., 2003), and start to enter the transplantation arena as shown by recent reports in mice, in which anti-C5 antibody treatment led to a complete block of terminal complement activity and prevented antibody-mediated rejection (Rother et al., 2006; Wang et al., 2007).

1.3. Role of Dxs as a complement inhibitor and EC protector

Low molecular weight dextran sulfate, a highly sulfated polysaccharide, was shown by Wuillemin et al. to act as a soluble complement inhibitor by enhancing the activity of C1 inhibitor (Wuillemin et al., 1997). Furthermore, Dxs binds the complement regulatory factor H (Pengaur et al., 1991). The polyanion Dxs therefore inhibits all three complement pathways as shown by hemolytic tests (CH50, AP50 and LP50) (Laumonier et al., 2003). In addition, it inhibits the coagulation system by enhancing the anticoagulatory activity of antithrombin III and C1 inhibitor against activated Factor XI (Wuillemin et al., 1996). Moreover, Dxs interferes with platelet adhesion (Zeelenber et al., 2003).

In our laboratory has shown that Dxs acts as an EC protector by protecting the EC from complement-mediated damage in allo- as well as xenotransplantation models and in myocardial I/R injury. This cytoprotective effect correlates with dose-dependent binding of Dxs to porcine and human EC. Binding of Dxs is considerably increased when EC are activated by complement or treated with heparinase. In addition, Dxs-bound to activated EC protects them from NK cell as well as complement-mediated cytotoxicity (Laumonier et al., 2003). Added to solution to whole human blood, hyperacutely xenograft rejection is attenuated in ex vivo pig lung perfusion with whole human blood (Banz et al., 2005). Dxs administered intravenously is viable in a heterotopic mouse-to-rat xenotransplantation model, in combination with cyclosporin, led to long-term xenograft survival (Laumonier et al., 2004). And as detailed below, we recently showed that Dxs facilitates anti-CD4 mAb-induced long-term allograft survival in rats despite of prolonged cold graft ischemia (Gajjanyake et al., 2008).

2. Dxs facilitates transplantation tolerance

The ultimate goal in clinical transplantation is to achieve enduring, donor-specific transplant tolerance, which appears to be a promising solution for the many drawbacks of immunosuppressive therapy. However, the translation of experimental immunological tolerance induction protocols, which work well in several animal models, to clinical setting has so far been unsuccessful. The establishment of tolerance is prevented by events which are inevitably associated with clinical transplantation, such as I/R injury and brain death of the organ donor. It was for example demonstrated that I/R injury prevents tolerance induction in experimental kidney transplantation (Coulsol et al., 2005), and consequently, it was recently highlighted that minimization of I/R injury associated with transplantation will be a key element in future tolerance induction strategies (Kang et al., 2007).

The mechanism by which I/R injury influences graft survival seems to be activation of the innate immune system. The latter plays an important role in recognition and elimination of pathogens through soluble proteins and membrane-bound receptors (Janeway and Medzhitov, 2002). Pattern recognition receptors (PRR), which are expressed on most of the cells in the immune system, not only recognize pathogens but are also capable of recognizing a number of endogenous ligands (Mellman, 2002). These endogenous ligands, are released as a consequence of cellular stress, which is commonly associated with I/R injury in organ transplantation (LaRosa et al.,
2007). In fact, strategies to inhibit innate immunity have previously been successful in experimental transplantation models. Use of the NF-κB inhibitor 15-deoxytetraspinone induced allograft tolerance in an anti-CD3 immunosuppressed non-human primate kidney transplantation model (Hutchings et al., 2003), and lack of MyD88 signaling induced long-term allograft survival in a mouse model (Walker et al., 2006), supporting the idea that inhibition of innate immunity provides a clinically relevant strategy to facilitate transplantation tolerance.

2.1. Role of complement in transplant tolerance

Activation of the complement system due to I/R injury helps bridging the innate immune response with the adaptive immune system and thereby influences the success of tolerance induction. Cytokines released as a result of triggering the innate immune system play an important role in breaking tolerance. It was shown that purified C3 and MBL, in combination with apoptotic blebs, modulated cytokines and upregulated IL-6 (Fraser et al., 2006). IL-6, in turn, appears to suppress the induction of regulatory T cells (Treg), which are important T-cell subsets associated with tolerance (Pasare and Medzhitov, 2003). Moreover, IL-6 and TGF-β lead to differentiation of T-cells into IL-17 producing Th17 cells and further suppress Treg induction (Chen et al., 2006).

Dempsey et al. showed that C3a functions as a molecular adjuvant of innate immunity (Dempsey et al., 1996). Ligation of C3b or C4b to antigen increased the antigen-specific proliferative and cytotoxic responses of T cells (Arvieux et al., 1988). Cleavage products of C3 and C4 bind antigen and regulate its processing and presentation at different levels. These cleavage products serve as ligands for the antigen and participate in its internalization and endosomal targeting as well as processing, suggesting augmented stimulation of antigen-specific immune response (Jacquelin-Safin et al., 1996). In contrast, ligation of complement receptor 3 (C3b, CD11b/CD18) by a cleavage product of C3, iC3b, modifies cutaneous CD11b+ cells such that skin antigen-presenting cells are unable to sensitize in a primary immune response, but actively induce antigen-specific tolerance (Hammerberg et al., 1998). Another study showed that iC3b engagement with CR3 on antigen-presenting cells (APC) in immune-privileged sites led to production of TGF-β2 and IL-10 required for induction of tolerance (Solari et al., 2003). In line with this finding, CD11b-knockout mice failed to develop oral tolerance upon antigen challenge (Ehrichtou et al., 2007). Hence, regulation of complement in tolerance induction appears to be an important strategy which influences the success of the treatment protocols.

2.2. Local complement production, activation and site-specific inhibition

The importance of local complement synthesis in allotransplantation has recently been highlighted by Sacks and Zhou (2005). The five most important differences of local versus systemic complement production are rapid action, tissue penetrability and site-specific regulation. Glomerular epithelial cells in the kidney spontaneously express the C3 gene and play a role in local, immune-mediated tissue injury (Sacks et al., 1993). Some of the complement factors, such as C3, have a large molecular weight (180kDa), which limits the availability of such molecules in the extracellular compartment, supporting the importance of local production for tissue penetrability (Sacks and Zhou, 2005). It was shown that expression of complement regulatory proteins was reduced in ischemic tissue, suggesting that not only production but also regulation of complement takes place site-specifically (Thurman et al., 2006). Therapeutic approaches targeted at systemic complement depletion may therefore not be efficient and are not desirable either, because complement contributes to microbial opsonization and immune complex clearing, and a lack of this function may render a graft recipient more prone to infections, especially when complement depletion is envisaged for a prolonged period of time and combined with immunosuppression. Indeed, a strategy targeted at systemic complement inhibition using the rodent C3 convertase inhibitor CR1-related protein γ-1g (Crry-1g) did not reduce renal I/R injury (Park et al., 2001). Strategies and/or substances which lead to site-specific, local complement inhibition are therefore clearly needed to prevent I/R injury in transplantation.

2.3. Effect of DXS in transplantation

DXS is a synthetic glycosaminoglycan containing approximately 12% of sulfur and has been investigated as a promising substance in experimental and preclinical studies for the past two decades. DXS serves as a potent complement inhibitor by inhibiting the classical, alternative and lectin pathway (Laumonier et al., 2003). Inhibition of the classical pathway by DXS is mediated through potentiation of C1-inhibitor (Wuillemin et al., 1997). C1-inhibitor binds to the C1s molecules and dissociates them from C1q. Other than potentiation of C1-inhibitor, DXS also binds to exposed throsters of activated C3 and C4 and thus inhibits their deposition (Wuillemin et al., 1997).

The exact mechanism leading to potentiation of C1-inhibitor by DXS is unknown (Thomas et al., 1995). However, sulfate groups seem to play a role since dextran, an alpha-1-4 linked polysaccharide similar to dextran, which is alpha-1-5 linked, has no anti-complement effect, whereas dextran bearing carboxylic and benzyl amide sulfonate groups inhibits complement activation in vivo in experimental models (Thomas et al., 1995, 1996). The interaction with the polyanion binding site on factor H has been proposed to be the mechanism by which DXS inhibits the alternative pathway of complement activation (Meri and Pangburn, 1994). DXS interacts with the factor H polyanion recognition site and by this enhances the binding of factor H to the C3b-target complex. The affinity of factor H to C3b is regulated at or near the 13th short consensus repeat (SCR) domain of factor H, which is a positively charged site.

In vitro investigation of DXS in a model of antibody-mediated cytotoxicity revealed that deposition of C1q, C3b and C4b from human serum on porcine aortic endothelial cells (PAEC) was dose-dependently inhibited (Laumonier et al., 2003). In addition, binding of DXS to human serum treated, and thus activated, but not to quiescent PAEC was observed using fluorescence labeled DXS. Moreover, DXS binding was favored by shedding of the natural EC-protective layer of heparan sulfate proteoglycans (HSPG) by pre-treatment of the cells with heparinase I. These findings prompted us to postulate a model which describes the ability of DXS to act as a "repair coat" to protect activated graft EC by, at least functionally, replacing the glycosaminoglycans on the cell surface.

Fig. 1. Model for DXS-mediated EC protection. (A) Cellular stress leads to EC activation and release of HSPG (green) from the cell surface. (B) "bare" (denuded) EC become a privileged target for molecules of innate immune activity, such as complement and NKG2 cells. (C) DXS (yellow) can act as a "repair coat" and protect EC from damage (from Laumonier et al., 2003).
cortex layer of the EC which was shed due to activation of the cells (Fig. 1). That the complement-inhibitory and EC-protective effect of DXS was indeed relevant also in vivo was proven later on in ahamster-to-rat xenotransplantation model, in which DXS injected into the recipient only during the first 2 weeks post transplantation, combined with continuous cyclosporin treatment, led to accommodation and long-term survival of the hamster xenograft (Laumonier et al., 2004). Binding of DXS to activated endothelium was also reported in a rat model of thrombotic microangiopathy (TMA) in the kidney. In this model, biotin-DXS staining was detected only on the surface of the glomerular endothelium with TMA but not in the kidney without TMA (Eto et al., 2005). These data suggest that DXS bears a unique capacity to bind to activated EC, thus conferring local, site-specific protection from complement-mediated damage.

![Graph showing cardiac allograft survival over time](image)

**Fig. 2.** Influence of DXS on anti-CD4 mAb induced tolerance induction. (A) DXS facilitates anti-CD4 (RIB 5/2) induced long-term graft survival after 12 h old graft ischemia time. DA-to-Leonc heterotopic rat cardiac xenograft transplantation was performed with 12 h of cold graft ischemia. Recipients were treated with either RIB 5/2 only or RIB 5/2+DXS. Kaplan–Meier graft survival curves are shown for both treatment groups. *p<0.05 vs compared with RIB 5/2 only. (B) (C) DA hearts subjected to 12 h of cold ischemia were transplanted into Lewis rats, which received RIB 5/2 with or without DXS. Recipients were sacrificed at day 1 after transplantation and the grafts immunostained for C3c and C9. Complement activity in serum was monitored by C3f50 assay after (C). a single dose of vs. injection of DXS (25 mg/kg) or (D) continuous treatment until day 5.
2.4. Effect of DKS on anti-CD4 mAb induced transplantation tolerance

Recently, we have demonstrated that combining DKS application with anti-CD4 mAb treatment facilitates tolerance induction in a prolonged graft ischemia model (Gajjalyale et al. 2008). In the used rat cardiac allotransplantation setting with anti-CD4 mAb treatment for induction of tolerance, extension of the cold graft ischemia time from 20 min to 12 h led to chronic rejection at around 50 days post transplantation, rather than establishment of tolerance. However, by treatment of the recipient with DKS for a period of 2 weeks, in addition to the anti-CD4 mAb therapy, allograft tolerance could be reestablished and graft survival prolonged to >100 days, which was indistinguishable to survival after only 20 min of cold graft ischemia. No difference in graft survival with and without DKS treatment was observed if the cold ischemia time was only 20 min. Furthermore, activation and damage of the graft endothelium was observed at 1 day post transplantation in the 12-h cold ischemia group but not in the 20-min ischemia group. Consequently, binding of DKS to the graft endothelium was only detectable after 12 h of cold ischemia. Complement deposition in graft tissue, detected as C3b/Cl in Clq, was significantly inhibited by DKS, whereas almost no systemic complement inhibition was observed by CH50 test (Fig. 2).

Early graft infiltration of granulocytes and monocytes/macrophages is known to boost the alloimmune response (Jassem et al. 2003; Moir et al. 2001), and we therefore investigated the effect of DKS treatment on graft infiltration by proinflammatory cells. In the 12-h ischemia groups DKS significantly inhibited graft infiltration by granulocytes and monocytes/macrophages, suggesting that also prevention of proinflammatory cell infiltration contributes to the beneficial effect of DKS. The mechanism of DKS-mediated inhibition of leukocyte infiltration in this model remains elusive. However, Matsumiya et al. showed earlier that dextran sulfate with 500,000 MW inhibits E-selectin mediated neutrophil adhesion to activated EC (Matsumiya et al. 1999). Another possible mechanism may be through inhibition of complement activation, which would reduce anaphylatoxin-mediated recruitment of inflammatory cells. Cleavage of C3 and C5 by convertases causes the generation of C5a and C5b, which possess potent chemotactic activity, particularly C5a. Cleavage of C5a by carboxypeptidase N into C5a des-arg preserves its chemotactic activity, which is not the case for C5a des-arg. It was also suggested that some isolated dextran may have immunomodulative properties, in particular that it inhibits the IFN-γ induced MHC class II expression on EC (shown for DKS 500,000 by Liu et al. 1996). Although soluble DKS serves as a competitive inhibitor of the binding of IFN-γ to membrane IFN-γ receptors, immobilized DKS can bind IFN-γ (Fernandez-Botran et al. 1999), suggesting that DKS has the capacity to bind immunoregulatory elements and subsequently target them to activated graft endothelium. However, the relative importance of DKS-mediated binding of immunoregulatory elements to the EC surface remains to be determined in separate experiments.

With respect to its effect on the coagulation system it was shown earlier that DKS prolongs the prothrombin time (PT), the activated partial thromboplastin time (aPTT) and the thrombin time (TT) (Zeelefelder et al. 2002). This could limit the clinical use of DKS because of the danger of bleeding complications. However, in our model DKS has had only a transient effect on coagulation in the treated graft recipients and administration of the indicated therapeutic doses did not cause major bleeding complications. We assume that the systemic anticoagulatory effect of DKS is limited in our model due to binding of the substance to the endothelium.

Whether a local anticoagulatory effect is adding to or even required for the beneficial effect of DKS on tolerance induction is unclear at the moment. In view of a clinical application of DKS or similarly acting substances the anticoagulatory effect needs to be considered, and further investigations on the need of anticoagulation to achieve the DKS-mediated attenuation of innate immunity are warranted.

In a recent phase I trial aPTT was used as an indirect measure of the DKS concentration. A systemic aPTT of 15 s, corresponding to a systemic DKS concentration of 30 μg/ml was achieved without any signs of increased bleeding. With heparin, an aPTT of 15 s is associated with severe bleeding risk. In fact, bleeding under DKS treatment is not associated with prolonged aPTT but rather with induction of thrombocytopenia, which occurs after about 3 days of continuous treatment (Frenz et al. 1991; Schmidt et al., in press).

3. DKS in islet allotransplantation

Ischemia/reperfusion injury is an important pathophysiological mechanism causing morbidity in many diseases and therapeutic modalities. In transplantation, IR injury is thought to contribute to both acute and chronic graft rejection. In addition to ischemia after procurement of the organ, the transition to brain death of the donor has been suggested to trigger oxidative stress, upregulate pro-inflammatory genes and influences the magnitude of IR injury in transplantation (Comerota et al. 2003; Pratschke et al. 2005). We have in a series of reports demonstrated that a thrombotic/inflammatory reaction is elicited in clinical islet transplantation when human pancreatic islets come in direct contact with ABO-compatible blood. This reaction is characterized by activation of the coagulation and complement systems, a rapid binding, and activation of platelets to the islet surface and infiltration of the islets by leukocytes, particularly granulocytes. We call this an instant blood-mediated inflammatory reaction (IBMIR) (Bennet et al. 1999). The IBMIR is the corresponding reaction to IR injury in islet cell transplantation, in which an induction of tissue factor (TF) and other inflammatory genes triggers this destructive thrombotic/inflammatory reaction (Bennet et al. 1999; Meberg et al. 2002; Pienini et al. 2002). Thus far, the IBMIR has only been linked to islet cell transplantation, but it is conceivable that similar reactions occur when isolated and cultured cells from other organs are infused into whole blood (e.g. hepatocytes, mesenchymal stem cells, T cells, dendritic cells), reflecting a more general problem in cell transplantation (Crennese et al. 2000).

In order to improve the outcome of clinical islet transplantation, the IBMIR needs to be attenuated. DKS has previously been shown to be a potent inhibitor of the activation of both the coagulation and the complement systems (Fiorente et al. 2001; Wailerem et al. 1997). Unlike high molecular weight dextran sulfate it does not activate the fibrinolytic system (Goto et al. 2004).

We have recently demonstrated the efficacy of DKS in preventing allogeneic and xenogeneic IBMIR in vitro and in vivo (Goto et al. 2004; Johansson et al. 2006). Most parameters reflecting the IBMIR, i.e. both the coagulation and complement cascades, platelet deposition, and infiltration of neutrophils, were attenuated in the animals treated with DKS compared to the controls. The effect of DKS on neutrophil infiltration is shown in Fig. 3. In the xenogeneic setting, T cell infiltration observed in some of the transplanted islet grafts was also effectively suppressed, demonstrating that the innate immune responses are attenuated by DKS. The improved morphological findings of intraportally transplanted islets correlated with a substantial prolongation of the survival of the transplanted adult porcine islets. Apart from the IBMIR, DKS has also been shown to have other positive effects, it potentiates the
mitogenic effect of hepatocyte growth factor on hepatocytes that might favor engulfment of the islets (Roos et al., 1995; Zornheck et al., 1995) and protect intrahepatic islet grafts in vivo (Nakano et al., 2000).

The exact mechanism by which DKS inhibits the IMMIR is unclear. Considering that IMMIR is triggered by TGFβ, it is likely that the previously identified agonistic effect on C1-inhibitor is able to mediate effects on the IMMIR. Unlike heparin, DKS does not contain any specific antithrombin binding sites. Despite this, DKS is far more effective in inhibiting the IMMIR than heparin, which needs to be combined with the complement inhibitor C1r/C1s in order to obtain any significant effect (Bennet et al., 1999). DKS, at concentrations between 2 and 20 μg/mL, has been shown to significantly reduce the risk of hyperacute rejection (HAR) of vascularized discordant xenografts (Florianie et al., 2001; Thomas et al., 1986), and DKS also prevented TF expression in a model of acute myocardial infarction (Benz et al., 2005). Complement activation plays a role in allogeneic IMMIR, but it occurs secondarily to coagulation (Bennet et al., 1999; Ozmen et al., 2002). This finding explains why complement activation is reduced in parallel with the reduction in thrombin-antithrombin complexes (TAT) at much lower concentrations (0.01–1 mg/mL) of DKS compared to those used in the HAR studies. Complement is activated during clotting by platelets which expose and release chondroitin sulfate upon activation. The chondroitin sulfate binds C1q and activates complement by the classical pathway (Hamad et al., 2008).

In a recently performed phase I study in normal individuals, we have shown that systemic DKS plasma concentrations up to 300 mg/mL (local intraportal concentration up to 50 mg/mL) can be reached without any side effects such as increased risk of bleeding (Schmidt et al., in press). This makes treatment with DKS during islet transplantation an attractive alternative.

4. Influence of DKS on dendritic cells

4.1. Interplay between complement, pattern recognition receptors and DKS

In 1994 Polly Matzinger postulated that the immune system evolved to respond not only to ‘foreign’ pathogens but also to ‘danger’ in the form of non-physiological cell death, damage or stress, and that both the ‘foreign’ and ‘danger’ signals are needed for induction of an adaptive immune response (Matzinger, 1994). Her group showed that DC can be activated by endogenous stimuli (Gallucci et al., 1999), and Shi et al. published later that cell injury leads to a release of an endogenous adjuvant, which enhances cytotoxic T cell responses (Shi et al., 2000). Molecules released from activated or dying cells, so-called damage-associated molecular patterns (DAMPs), can then lead to a phenomenon called “sterile inflammation”. Similar to pathogen-associated molecular patterns (PAMPs), DAMPs are recognized by PRR such as toll-like receptors (TLR), NOD-like receptors, RIG-like helicases or the receptor of advanced glycation end products (LaRosa et al., 2007). As recently reviewed by (Xeno and Rock, 2008), DAMPs include a wide range of molecules like heat shock proteins, HMGB1, ATP, uric acid, heparan sulfate, hyaluronan, etc. They can trigger the release of pro-inflammatory mediators, including cellular molecules which activate the complement system. It was for example shown, that non-muscle myosin released from dying cells is recognized by naturally occurring IgM antibodies, resulting in complement activation and tissue damage (Zhang et al., 2006a). Furthermore, it has been demonstrated, that lipo injury leads to exposure of new epitopes on EC, which are also recognized by natural IgM and activate complement via the lectin pathway (Zhang et al., 2006b).
The endothelial surface layer, the glycocalyx, is composed of glycoproteins and glycolipids, among which HSPG are very abundant. Major cell surface membrane HSPG can be subdivided into syndecans (four members) and glypicans (six members), as well as several minor membrane HSPG like epican or betaglycan (Esko and Lindahl, 2001). The HSPG layer of healthy EC is crucial for the anticoagulant and anti-inflammatory properties of the endothelium, by enhancing the activity of tissue factor pathway inhibitor (TFPI) and antithrombin III (de Agostini et al., 1990; Marcum et al., 1986; Rosenberg and Rosenberg, 1984). Quiescent EC express the ectoenzyme nucleoside triphosphate diphosphohydrolase (NTPDase, CD39), which degrades platelet-derived ATP and ADP (Kaczmarek et al., 1996). Moreover, HSPG bind superoxide dismutase, which is responsible for the degradation of reactive oxygen species (ROS) (Karlsson et al., 1988). In addition, HSPG are involved in almost every stage of leukocyte transmigration through the blood vessel wall, with heparan sulfate (HS) acting as a ligand of E-selectin (Wang et al., 2005) and CR3 (Diamond et al., 1995). Furthermore, several chemokines have been shown to interact with HS on the surface of the cells as well as in the extracellular matrix (Handel et al., 2005).

HSPG are rapidly released from the EC surface under conditions of inflammation and tissue damage (Birke and Platt, 1996; Platt et al., 1990, 1991). Just recently this has also been shown in the plasma of vascular surgery patients (Rehm et al., 2007), in which elevated levels of syndecan-1 and HS was found just 15 min after reperfusion. HS release is supposed to be mediated by proteolytic cleavage of the protein core or by endoglycosidic cleavage of the HS chains (Birke et al., 1994; Birke and Platt, 1996). Free HS has been shown to act as an endogenous ‘danger’ signal by inducing maturation of macrophages and DC via TLR4 (Johnson et al., 2002; Tang et al., 2007; Wrenshall et al., 1995, 1999). Mature DC are characterized by strongly reduced phagocytic activity as well as upregulated antigen-presenting and T cell costimulatory molecules. Moreover, mature DC secrete predominately pro-inflammatory cytokines such as IL-1β, IL-6, IL-12/70 and TNF-α (Rangereau and Steinman, 1998).

TLR2 and TLR4 have been shown to be crucially involved in LPS mediated inflammatory responses in kidney (Leemans et al., 2005; Wu et al., 2007) and heart (Kaczorowski et al., 2007). Kaczorowski et al. showed in a murine cardiac transplanation model that serum levels of TNF-α, IL-1β, IL-6, proinrin I and MCP-1 were dramatically reduced in mice deficient in TLR4 signaling. Furthermore, these mice had reduced intragraft mRNA levels of TNF-α, IL-1β, IL-6, EGR-1, ICAM-1 and iNOS (Kaczorowski et al., 2007). Recent publications also described a critical interplay between TLR and complement after co-activation in vitro. Haulich demonstrated a regulatory role of C5a on TLR4 induced immune responses in vitro and in vivo. C5a has been shown to negatively regulate production of IL-12 family members such as IL-12, IL-23 and IL-27 in inflammatory macrophages (Haulich et al., 2005). Mice deficient in the membrane complement inhibitor DAF have elevated levels of TNF-α, IL-1β and IL-6 in response to TLR agonists, whereas the levels of IL-12/40 are slightly decreased (Zhang et al., 2007).

In our laboratory, we have investigated the effect of DKS on the maturation process of human DC in vitro in response to endogenous or exogenous TLR agonists as well as pro-inflammatory cytokines (Sprig et al., 2008). DKS is able to prevent phenotypic maturation of human monocyte-derived DC as well as freshly isolated peripheral myeloid DC ex vivo. Upregulation in response to HS, LPS (both TLR4) or LTA (TLR2) of co-stimulatory molecules like CD80, CD86 and CD40 are inhibited by DKS in a dose-dependent manner, and secretion of the pro-inflammatory cytokines TNFα, IL-6 and IL-1β is reduced. In addition, antigen-presentation and T cell proliferation was reduced by DKS. Interestingly, however, DKS does not prevent the decrease of phagocytic activity during TLR induced maturation.

Furthermore, the impact of DKS on TLR signaling in DC was examined. We found that DKS significantly inhibits the phos-

Fig. 4. DKS inhibits phenotypic and functional maturation of human DC induced by endogenous and exogenous TLR agonists in vitro. (1) Upregulation of costimulatory and adhesion molecules is inhibited by DKS. (2) DKS prevents the release of proinflammatory cytokines in response to TLR triggering. (3) Phosphorylation of IκB-α is prevented which results in reduced release of activated transcription factor NF-κB. (4) Treatment of DC with DKS and a TLR ligand results in a decrease of C1q secretion whereas more C1 is released.
Phosphorylation of IkBα after 15 and 30 min of stimulation by TLR ligands. Phosphorylation of IkBα leads to its ubiquitination and subsequent degradation, which results in the release of the pro-inflammatory transcription factor NF-κB. Activation of NF-κB was strongly prevented by DKS. In summary, the recently published data show that DKS not only acts as a complement inhibitor and EC protectant, but also directly influences the crosstalk between innate and adaptive immunity by preventing DC maturation. DKS and similarly acting, non-toxic inhibitors of complement and TLR activation may therefore be of great therapeutic interest for the prevention of I/R injury, particularly in the context transplantation (Fig. 4).

4.2. DKS, DC complement proteins and tolerance

The balance between the induction of immune reactivity and immune modulation, including induction of tolerance, seems to be determined by the subtypes of the DC, their state of maturation, and the secretion of soluble mediators including cytokines. Similar to macrophages, immature DC have been described as a rich source of the complement proteins C1q (Castellan et al., 2004; Kaul and Loos, 2001), C3 (Reis et al., 2006), C4BP, C7, C8, Factor I and Factor B (Reys et al., 2006), (2007) in mice and humans. C1q produced by immature DC is functionally active in complement activation and binding to apoptotic cells and is necessary for clearance of the latter (Castellan et al., 2004; Nauta et al., 2003, 2004). The production of C1q is down-regulated upon DC maturation in vitro and in vivo (Castellan et al., 2004). Recently, the effect of C1q on differentiation of monocytes into immature DC was examined. DC differentiated in the presence of C1q secrete less TNF-α, IL-6 and IL-10 in response to LPS. In addition, upregulation of CD80, CD83 and CD86 was impaired along with a reduced capacity to stimulate allogeneic T cells (Castellan et al., 2007).

DC also express several complement receptors such as the CR3 and complement receptor 4 (CR4, CD11b/CD18) as well as the seven-transmembrane G-protein coupled receptors CCR3 and C5aR. Binding of iC3b to CR3 on antigen-presenting peritoneal exudate cells (PEC) leads to an increase of IL-10 and TGF-β secretion in vitro (Sohn et al., 2003). CR3 and CR4 are supposed to be apoptotic-cell receptors, and signaling through these receptors has been shown to render human monocyte-derived DC tolerogenic (Skoberne et al., 2006). We examined whether DKS binds to CR3 on DC in vitro, as shown before for heparin (Peter et al., 1999), another well known sulfated glycosaminoglycan. Blocking of CR3 with the chimeric monoclonal antibody c7E3 did not reverse the inhibitory effect of DKS, nor the binding of DKS-fluo to immature DC (Spirig et al., 2008). The inhibition of DC maturation observed by DKS therefore does not seem to be due to interaction of the substance with CR3.

On the ligand side, C3 synthesis by murine DC was reported to be essential for T cell activation: C3 deficient murine DC have a reduced potency to stimulate allogeneic CD4+ T cells in vitro and in vivo and drive the immune response towards a Th2 phenotype characterized by low levels of IFN-γ and high levels of IL-4 (DiLillo et al., 2006). C3 also seems to influence the differentiation of human monocytes into immature DC. Monocytes differentiated in C3 deficient serum express lower levels of costimulatory molecules CD80/CD86, lower levels of DC-SIGN, MHC class II and CD1a (Reis et al., 2008). In our own studies we could show that DKS decreases the secretion of C1q, whereas the production of C3 was increased when cells where treated together with a TLR stimulus (Spirig et al., 2008). C3 production in human monocytes seems to be regulated by transforming growth factor-β2 (TGF-β2) in vitro. C3 production on the protein as well as on the mRNA level was induced for up to 72 h (Dreyfus et al., 2006). The induction has been shown to be mediated by protein kinase C-dependent (PKC) pathway (Dreyfus et al., 1998). Furthermore, proinflammatory cytokines like IL-1α and IFN-γ and the combination of IL-1β and IL-6 lead to an increase of C3 synthesis by human hepatoma-derived cell line (HepG2) (Andrews et al., 2003). It therefore needs to be examined whether the elevated levels of C3 in our setting are due to elevated TGF-β2 or IL-1α production of these cells.

5. Conclusions

As summarized in Fig. 5, DKS develops its anti-inflammatory properties by protecting the endothelium of the donor graft from complement-mediated damage. In addition, as shown in vivo, DKS seems to have an inhibitory effect on TLR induced maturation of

![Fig. 5. Interplay between DKS, endothelium, DC and complement. (1) The cell surface of a healthy endothelium is covered by a layer of HS PC. (2) I/R injury leads to activation of the complement system. (3) Activation of the EC leads to shedding of HS PC and their heparin sulfate chains (HS) and other molecules act as damage-associated molecular patterns (DAMPs) which are recognized by PRR. (4) DAMPs are recognized by DC via PRR, which leads to maturation of the cells. DKS has been shown to prevent both complement mediated IC damage and maturation of human DC.](image-url)
human DC. Results obtained in an anti-CD4 mAb-induced transplantation tolerance model support our hypothesis, that DCS exerts its protective effect not only on the vasculature, but in addition influences the activation of innate immune cells as, e.g. maturation of DC or the migration behavior of granulocytes. Furthermore, in islet transplantation DKS was shown to prevent BMR in vitro and in vivo.

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3 Discussion

The process of ischemia/reperfusion injury is multifactorial in origin and detrimental to the outcome of allograft survival. If proper care is not applied to minimize the I/R injuries, the outcome for the allograft is poor, particularly if transplantation tolerance should be achieved. Recently, Kang et al. (98) has proposed that minimization of I/R injury is a key factor in tolerance induction; therefore, the strategies aimed at reducing I/R injury are important in transplantation tolerance. However, at present there is no definitive treatment available to prevent I/R injury in the clinical setting.

The present work is primarily concerned with the protection of the vascular endothelium as a therapeutic intervention in transplantation and other vascular surgical interventions. Low molecular weight dextran sulfate, well known as EC protectant, was used as a candidate to attenuate I/R-mediated injuries.

3.1 Minimization of I/R injury facilitates tolerance

It has been proposed that the organs retrieved from cadaver donors with substantial cold ischemia time exhibited severe post-transplantation inflammation, which jeopardizes the allograft (131). The strategies aimed at reducing I/R injury-mediated activation of the innate immunity showed a long-term allograft acceptance in experimental models (99, 100).

In the present study, the therapeutic approaches targeting the innate immunity using DXS in combination with the non-depleting anti-CD4 mAb RIB 5/2 significantly extended the survival of the allograft, which is transplanted after prolonged cold ischemia. It is plausible to induce allograft tolerance with short ischemic time; presumably short ischemic time minimizes the organ damage. This has been confirmed in our model, in which RIB 5/2 per se led to prolonged allograft survival, with more than 175 days of graft survival and good histology. However, the same treatment in the 12-h ischemia group led to chronic rejection, characterized by inflammatory cell infiltration, intimal thickening and parenchymal fibrosis, which are the typical features of cardiac allograft vasculopathy (CAV). Treatment with DXS together with the tolerance induction protocol in the 12-h ischemia group prolonged graft survival compared with RIB 5/2 alone. Analysis of long-term grafts revealed a well-preserved cardiac architecture and the absence of ongoing inflammation.
3.2 Effect of DXS on the innate immunity

Activation of the complement system and subsequent complement-mediated tissue damage has been greatly considered in I/R injury. Although DXS had almost no systemic effect on the complement system in our experiments, it significantly reduced local complement deposition at 1 d post-transplantation. In line with recent evidence, highlighting the importance of local complement production (132), our results indicate that local inhibition of complement activation may protect the allograft during tolerance induction phase and therefore facilitate the latter. Moreover, early graft infiltration by granulocytes and monocytes/macrophages critically influences the long-term alloimmune response (133, 134). Therefore, it is tenable that the observed inhibition of granulocyte and monocytes/macrophages infiltration by DXS is positively correlated with the long-term graft survival. Matsumiya et al. (113) showed that DXS inhibits E-selectin-mediated neutrophil adhesion to activated EC. Furthermore, DXS may attenuate anaphylatoxin-mediated recruitment of inflammatory cells through inhibiting complement activation. Consistent with this observation, it has been also suggested that DXS has immunosuppressive properties. DXS was shown to inhibit the IFN-γ-induced MHC-II expression on endothelial cells (117). Although soluble DXS serves as a competitive inhibitor of the binding of IFN-γ to membrane IFN-γRs, immobilized DXS can bind with IFN-γ (135). This suggests that DXS has the capacity to bind immunoregulatory elements and subsequently target them to activated graft endothelium. However, the relative importance of DXS-mediated binding of the immunoregulatory elements to EC surface remains to be determined in a separate experiment.

As would be expected because DXS is an anticoagulatory substance (111), it has a transient effect on coagulation in the treated graft recipients. However, administration of the indicated dose of DXS in our model did not cause any major bleeding complications. It is as yet unclear whether or not this anticoagulatory effect is beneficial for long-term graft acceptance. In view of a clinical application of DXS or similarly acting substances, the anticoagulatory effect needs to be considered, and further investigations on the need of anticoagulation to achieve the DXS-mediated attenuation of innate immunity are warranted. Generation of anti-dextran sulfate antibodies, which might limit the clinical use of DXS, has not been described following i.v. administration so far, despite the quite extensive use of DXS in different animal models as well as preclinical studies (103, 112, 136).

It has been reported that the use of FTY720 in combination with RIB 5/2 prevents tolerance induction (137); therefore, the selection of drugs for synergistic treatment with tolerance induction protocols appears to be a challenge. Our novel approach of graft-targeted treatment by DXS might thus offer new, clinically relevant perspectives
Discussion

to attenuate innate immunity in the context of tolerance induction. We showed that prolonged cold graft ischemia for 12 h, but not for 20 min ischemia, critically influenced damage and activation of the graft endothelium, characterized by the shedding of HSPG. As a consequence, the graft endothelium seems to become a target for binding of DXS, which then reestablishes its anti-inflammatory and anticoagulatory properties. In other words, DXS reinstates the 'non-dangerous' (59) properties of the graft endothelium. Using a fluorescence-labeled variant of DXS, DXS-Fluo, we could indeed observe binding of DXS to graft endothelium after 12 h of cold ischemia, but not after 20 min. As shown earlier by others and ourselves (102, 104), binding of DXS was inversely correlated to the endothelial expression of HSPG.

In another in vivo study using an infrarenal aortic clamping model in rats, we showed that DXS attenuates I/R-induced EC activation, which is characterized by loss of EC integrity and an enhanced pro-inflammatory environment. In this study, DXS influenced the mitogen activated protein kinase (MAPK) pathway, particularly JNK and ERK1/2 signaling. Activation of the MAPK pathway has been shown to be detrimental for complement mediated injuries and activation of ERK1/2 plays a major role in terminal complement component, C5b-9-induced proliferation of differentiated human aortic smooth muscle cells (ASMC) in culture (138). Complement-induced ERK activation depends on cytoskeletal remodeling and affects the regulation of distinct downstream substrates, while chronic, constitutive ERK activation exacerbates complement-mediated glomerular epithelial cell (GEC) injury (139). Complement C5b-9 activates c-Jun N-terminal kinase (JNK) in cultured rat GECs and that JNK activity is increased in glomeruli isolated from proteinuric rats with passive Heymann nephritis, as compared with control rats (140). Inhibition of MAPK activation, particularly JNK and p38-MAPKs has been shown to reduce injury, also complement-mediated, in various target organs and models (141, 142). Therefore, DXS-mediated downregulation of MAPK signaling is beneficial in reducing I/R injury. However, the exact mechanism of DXS-mediated downregulation of JNK and ERK1/2 remains elusive. We could not exclude that this might be due to direct inhibition of signaling or as a consequence of complement inhibition. However, it has been shown that the interferon gamma-induced Jak-Stat pathway is inhibited in human vascular endothelial cells (118) and NFk-b translocation in DC (91). Therefore, it is tenable to design experiments to investigate the exact effect of DXS on signaling pathways. To date, however, it remains unknown whether complement inhibition and accompanying modulation of MAPK signaling may be beneficial in reducing I/R-induced tissue injury. Our results indicate that complement inhibition with DXS is associated with reduced JNK and ERK1/2 activation.
3.3 Monitoring tolerance

Monitoring the tolerance state of patients appears to be essential in understanding the success or failure of the clinical transplantation tolerance. Expression of genes related to tolerance or rejection has been highlighted experimentally and clinically (143, 144). Analysis of intragraft gene expression at 40 d and 100 d in the rat models of our experiments revealed a significant upregulation of the gene TOAG-1 at both time points, consistent with the recent finding that TOAG-1 gene is specifically and reproducibly upregulated in the long-term surviving allografts (143). The observed upregulation of genes such as CD25, CD3, and CD40L in long-term surviving grafts compared with rejected grafts plays a dominant role in maintaining tolerance (145, 146). Moreover, elevation of proinflammatory related genes does not necessarily reflect the rejection rate (144, 145). However, finding the clinically valuable genes, which can be used to monitor tolerance, is being investigated extensively experimentally and clinically.

Another surrogate marker for tolerance monitoring is the analysis of allo-specific antibody. In the primate tolerance induction model, monitoring anti-donor antibody by flow cytometry appears to be a useful assay to monitor tolerance (147). In our experiments, chronic graft rejection was linked to a pronounced IgM and IgG alloantibody response, which was absent in RIB 5/2+DXS-treated recipients with long-term surviving allografts. This finding is in line with recent reports (146), and we conclude that inhibition of the early innate immune response by DXS treatment may play an important role in lowering the anti-graft IgG and IgM response, thus preventing chronic rejection.

3.4 Limitations of LC-MS/MS-based hepcidin analysis

Although several methods have been described based on mass spectrometry, our preliminary data propose that quantification of serum hepcidin in clinical samples might be limited in mass spectrometer based methods. It has been previously considered that hepcidin has strong non-specific binding capacity to surfaces during sample preparation and this appears to result in lower recoveries in LC-MS/MS based methods (148). We have experienced that non-specific binding of hepcidin during sample preparation may lead to considerable loss after LC-MS/MS analysis.

Moreover, oxidation of hepcidin is another condition that appears to limit quantification in LC-MS/MS based methods. Adding dithiothreitol (DTT) to the samples from the very beginning of the experiments appears to be a good strategy; however, it did not prevent completely oxidation of hepcidin. Therefore, non-specific
binding of hepcidin and oxidation during sample preparation appear to be the major barriers to limit quantification of LC-MS/MS based methods. Hence, our finding allows improving new sample preparation methods in spectrometer-based analysis of hepcidin in serum.

3.5 Conclusions

In conclusion, our results suggest that dextran sulfate attenuates acute graft injury related to prolonged ischemia time. This strategy helps to enhance tolerance induction in our animal model. We provide evidence that targeted cytoprotection of activated/damaged endothelium, using EC protectants such as dextran sulfate, may functionally restore the anti-inflammatory and anticoagulant properties of the luminal glycocalyx. This may provide a novel way of attenuating reperfusion injury in the clinical setting.

3.6 Outlook and Perspective

The endothelial cell protectant low molecular weight dextran sulfate (DXS), used in our experiments, revealed that it has a unique capacity to bind to vascular endothelium, which is activated after ischemia. Endothelial cell protection with DXS leads to attenuation of the innate immune response and facilitates the tolerance induction protocols. Spirig et al. (91) showed that DXS prevents maturation of DC in vitro. Dendritic cells, which have a tolerogenic capacity, play an important role in transplantation tolerance. Therefore, the effects of DXS on DC maturation in clinically relevant animal models, such as the prolonged ischemia model, may help to elucidate an additional therapeutic value.

Establishment of a clinically more relevant model to test transplantation tolerance is an important factor in understanding the mechanism of tolerance. Today, most of the animal experiment models used are young and healthy donors with no prolonged ischemia or brain death to test tolerance induction protocols. This is different from the real clinical transplantation, in which I/R injury and brain death are common factors. Hence, the innate immune responses are augmented by I/R injury and/or brain death at the early stage, creating resistance to inducing tolerance. Therefore testing the tolerance induction capacity in a combination model of prolonged ischemia and brain death might be more clinical relevant.

Moreover, analysis of tolerance success/failure genes at the early stage would help to establish a gene expression profile for the prediction of transplantation tolerance.
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5 List of Publications

Original papers and peer-reviewed review


Abstracts


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  - Isolated heart perfusion methods
  - Cell isolation
  - Isolated blood vessels in organ chamber experiments
- Immunofluorescence, immunohistochemistry and histology techniques
- Light and fluorescence microscope (Nikon Eclipse TE2000-U) & LSM confocal microscope
- Electron microscopy
- SDS-gel electrophoresis
- Western blot Analysis - ChemiDoc™ XRS and The Odyssey® Infrared Imaging System
- Flow Cytometer (BD FACScan flow cytometer / FlowJo)
- ELISA, aPTT test and CH-50 test
- Solid phase extraction (SPE) for LS MS/MS (Oasis™ HLB Sample extraction)
- Molecular biology techniques

Professional Experience

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7 Declaration of Originality

Last name, first name: Gajanayake Thusitha

Matriculation number: 03-217-957

I hereby declare that this thesis represents my original work and that I have used no other sources except as noted by citations. All data, tables, figures and text citations, which have been reproduced from any other source, including the internet, have been explicitly acknowledged as such. I am aware that in case of non-compliance, the Senate is entitled to divest me of the doctorate degree awarded to me on the basis of the present thesis, in accordance with the “Statut der Universität Bern (Universitätsstatut; UniSt)”, Art. 20, of 17 December 1997.

Place, date

Bern, 02-01-2009

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