Genetic, Physiological, and Pharmacological Amelioration of Ischemic Injury

Denis Susa

Genetic, Physiological, and Pharmacological Amelioration of Ischemic Injury
Genetische, fysiologische en farmacologische bescherming tegen ischemische schade

Thesis, Erasmus MC Rotterdam, with a summary in Dutch
ISBN: 978-90-9025695-5

© D. Susa, 2010. No part of this book may be reproduced, stored in a retrieval system, in any form or by any means, without the written permission of the author or from the publisher holding the copyright of the published articles.

Cover and lay-out design by Dare to Design*, Rotterdam.
Printed by Ridderprint, Ridderkerk

Financial support for the publication of this thesis was provided by:
Astellas Pharma BV, R.W. Kluin, Nutricia Nederland BV, Biomet BV, J.E. Jurriaanse Stichting, Covidien Nederland BV

Genetic, Physiological, and Pharmacological Amelioration of Ischemic Injury

Genetische, fysiologische en farmacologische
bescherming tegen ischemische schade

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam

op gezag van de
rector magnificus
Prof.dr. H.G. Schmidt

en volgens besluit van het College voor Promoties.
De openbare verdediging zal plaatsvinden op
3 november 2010 om 11.30 uur

door

Denis Susa
geboren te Rotterdam

PROMOTIECOMMISSIE

Promotor:	Prof.dr. J.N.M. IJzermans
Overige leden:	Prof.dr. J.H.J Hoeijmakers
	Prof.dr. D.J.G.M. Duncker
	Prof.dr. R. Benner
Copromotor:	Dr. R.W.F. de Bruin

CONTENTS

Chapter 1	Introduction and aim of the thesis	7
Chapter 2	Mechanisms of ageing in chronic allograft nephropathy *Journal of Organ Dysfunction 2007; 3 (1): 7-17*	13
Chapter 3	Congenital DNA repair deficiency results in protection against renal ischemia reperfusion injury in mice *Aging Cell 2009; 8 (2): 192-200*	33
Chapter 4	Long-term results after renal ischemia reperfusion injury in DNA repair deficient mice	49
Chapter 5	On the role of adenosine in classical and interorgan preconditioning of the heart *In: Myocardial Ischemia and Preconditioning, edited by NS Dhalla, N Takeda, M Singh, A Lukas, Chapter 22: 317 – 328. Kluwer Academic Publishers, Boston 2003*	59
Chapter 6	Amelioration of renal ischemia reperfusion injury by synthetic oligopeptides related to human chorionic gonadotropin *Nephrology, Dialysis and Transplantation 2009; 24 (9): 2701-8*	71
Chapter 7	Donor pre-treatment with tacrolimus reduces transplant vasculopathy *Pharmacological Research 2009; 59 (4): 273-8*	87
Chapter 8	Differential expression of inflammatory, cytoprotective and injury genes in kidneys of heart beating and non heart beating donors *Submitted*	101
Chapter 9	Summary and general discussion	115
	Nederlandse samenvatting	123
	Dankwoord	127
	Curriculum Vitae	129

Chapter 1

Introduction

INTRODUCTION

Organ transplantation is the treatment of choice for end stage organ failure, and in recent decades one year results have greatly improved. Two major problems facing the transplant community remain; long-term survival of organ grafts has still not reached its full potential, and the shortage of suitable donor organs is still increasing [1].

Long-term graft survival is significantly hampered by chronic transplant dysfunction (CTD). This still poorly defined process of untreatable functional deterioration of an organ following transplantation accounts for approximately 30 percent of graft loss in the first 5 years after transplantation. In the kidney CTD, also known as chronic allograft nephropathy (CAN), is clinically characterized by progressive renal dysfunction, associated with hypertension and proteinuria [2]. Renal biopsies show characteristic but nonspecific histopathological changes in the vascular, glomerular and tubulointerstitial compartments of the kidney, which overlap with the histology of normally aging kidneys. Although the term CAN has been recently replaced by "interstitial fibrosis and tubular atrophy without evidence of any specific etiology" [3], we continue to use CAN in this thesis, as this is the terminology used in the majority of the literature available to date.

Increasing evidence suggests that the development of CAN is multifactorial in origin, with allo-antigen dependent and allo-antigen independent factors contributing to the decline of renal graft function [4-6]. Of the allo-antigen independent factors, donor age and cold ischemia time seem to be the major correlate to transplant survival [7, 8]. Clinical observations suggest an inverse relationship between both donor age and ischemia time and long-term graft survival [9-11]. The mechanisms by which donor age or ischemia lead to reduced functional life span are currently unknown.

Several studies have shown the accelerated appearance of aging-phenotypes in transplanted organs, suggesting that mechanisms of aging may operate at an accelerated pace in the development of CAN [12-14].

Reactive oxygen species (ROS) are produced in excess during ischemia reperfusion after transplantation and are a primary source of cellular damage. Accumulation of oxidative DNA damage generated by ROS formed as a consequence of cellular metabolism is also considered one of the major reasons why we age [15]. This suggests that normal aging, ischemia reperfusion injury and long-term organ rejection may share a common mechanistic basis.

Due to the growing shortage of donor organs, alternative sources of organs are increasingly explored. Grafts from so-called marginal, or expanded criteria donors (ECD) are increasingly used for transplantation. This source includes organs from donors > 60 years of age, donors with hypertension and other pre-existing disease. Although recipients of a marginal kidney have a survival benefit compared to candidates who remain on the waiting list for transplantation, graft function and graft and patient survival following transplantation of a marginal organ are worse compared to transplantation of organs from standard criteria donors [16, 17]. Reasons for this observation are that graft outcome is adversely affected by donor age, and associated risk factors such as pre-existing disease. Moreover, old organs are more vulnerable to the adverse effects of brain death and ischemic injury. Nevertheless, the number of ECD kidney donors has increased substantially

over the last years [18].
Protection of ECD grafts through amelioration of ischemic, surgical and inflammatory damage, may improve graft function and survival of those grafts on the long-term. Until now, several methods have been introduced to prevent ischemic injury per se with varying results. Of these, the induction of ischemic tolerance seems to be rather promising. The concept of ischemic tolerance is founded on the premise that a brief period of low-grade ischemic injury protects against subsequent prolonged exposure to ischemic injury [19]. In addition to classic ischemic pre-conditioning, several compounds are also able to induce ischemic tolerance, a phenomenon called "pharmacological pre-conditioning".

Aim of the thesis

In the first part of this thesis, we aimed to elucidate the role of oxidative DNA damage and repair in the process of renal ischemia reperfusion injury and chronic allograft nephropathy. We have used knockout mice that are defective in DNA repair pathways to determine the consequences of defective repair of oxidative lesions for the development of renal ischemia reperfusion injury.
We addressed whether these pathways, whose absence results in accelerated aging syndromes, could affect acute and chronic outcome using a renal ischemia reperfusion injury model in mice.
In the second part of this thesis, we explored three new treatment options to combat ischemia reperfusion injury and CTD. First, we investigated the protective role of adenosine and its receptors A_1 and A_3 in ischemic preconditioning in the rat heart. Although a role for adenosine in distant ischemic preconditioning has been postulated, the mechanism underlying inter-organ preconditioning remains elusive. We therefore tested the hypothesis that adenosine released by the remote ischemic organ stimulates local afferent nerves, which leads to activation of myocardial adenosine receptors, resulting in protection of the myocardium.
Next, we investigated the immunoregulatory properties of oligopeptides related to the β-chain of the pregnancy hormone ß-chorionic gonadotropin (ß-hCG). It has been previously reported that small synthetic oligopeptides related to human ß-hCG can reduce inflammation in a sepsis model. Here we tested whether such oligopeptides can reduce renal ischemia reperfusion injury in the mouse.
Further, we explored the possibility to use common immunosuppressive agents to pre-treat organ donors with the aim to protect the organ against the consequences of ischemic -, and surgically induced injury on transplant vasculopathy.
To understand the superior long-term survival of kidneys from living donors (LD) compared to brain death (BD) and (NHB) donors, we investigated the impact of brain dead and asystole by performing comparative studies on transcriptional changes that occur after organ retrieval and cold storage in kidneys from NHB, BD and LD donors. To this end, expression levels of genes indicative of inflammation, cytoprotection, and injury after clinically relevant cold ischemia times were examined.
The experimental data of the various studies have been discussed in the context of literature data in Chapter 9, the Summary and general discussion. In this chapter also suggestions are given for future research in this area.

REFERENCES

1. LECHLER, R.I., ET AL., ORGAN TRANSPLANTATION--HOW MUCH OF THE PROMISE HAS BEEN REALIZED? NAT MED, 2005. 11(6): P. 605-13.
2. HALLORAN, P.F., CALL FOR REVOLUTION: A NEW APPROACH TO DESCRIBING ALLOGRAFT DETERIORATION. AM J TRANSPLANT, 2002. 2(3): P. 195-200.
3. SOLEZ, K., ET AL., BANFF '05 MEETING REPORT: DIFFERENTIAL DIAGNOSIS OF CHRONIC ALLOGRAFT INJURY AND ELIMINATION OF CHRONIC ALLOGRAFT NEPHROPATHY ('CAN'). AM J TRANSPLANT, 2007. 7(3): P. 518-26.
4. LI, C. AND C.W. YANG, THE PATHOGENESIS AND TREATMENT OF CHRONIC ALLOGRAFT NEPHROPATHY. NAT REV NEPHROL, 2009. 5(9): P. 513-9.
5. NANKIVELL, B.J. AND J.R. CHAPMAN, CHRONIC ALLOGRAFT NEPHROPATHY: CURRENT CONCEPTS AND FUTURE DIRECTIONS. TRANSPLANTATION, 2006. 81(5): P. 643-54.
6. PASCUAL, M., ET AL., STRATEGIES TO IMPROVE LONG-TERM OUTCOMES AFTER RENAL TRANSPLANTATION. N ENGL J MED, 2002. 346(8): P. 580-90.
7. TERASAKI, P.I., J.M. CECKA, AND D.W. GJERTSON, IMPACT ANALYSIS: A METHOD FOR EVALUATING THE IMPACT OF FACTORS IN CLINICAL RENAL TRANSPLANTATION. CLIN TRANSPL, 1998: P. 437-41.
8. KANEKU, H.K. AND P.I. TERASAKI, THIRTY YEAR TREND IN KIDNEY TRANSPLANTS: UCLA AND UNOS RENAL TRANSPLANT REGISTRY. CLIN TRANSPL, 2006: P. 1-27.
9. MIKHALSKI, D., ET AL., COLD ISCHEMIA IS A MAJOR DETERMINANT OF ACUTE REJECTION AND RENAL GRAFT SURVIVAL IN THE MODERN ERA OF IMMUNOSUPPRESSION. TRANSPLANTATION, 2008. 85(7 SUPPL): P. S3-9.
10. MORRIS, P.J., ET AL., ANALYSIS OF FACTORS THAT AFFECT OUTCOME OF PRIMARY CADAVERIC RENAL TRANSPLANTATION IN THE UK. HLA TASK FORCE OF THE KIDNEY ADVISORY GROUP OF THE UNITED KINGDOM TRANSPLANT SUPPORT SERVICE AUTHORITY (UKTSSA). LANCET, 1999. 354(9185): P. 1147-52.
11. SALAHUDEEN, A.K., N. HAIDER, AND W. MAY, COLD ISCHEMIA AND THE REDUCED LONG-TERM SURVIVAL OF CADAVERIC RENAL ALLOGRAFTS. KIDNEY INT, 2004. 65(2): P. 713-8.
12. FERLICOT, S., ET AL., THE ROLE OF REPLICATIVE SENESCENCE IN CHRONIC ALLOGRAFT NEPHROPATHY. HUM PATHOL, 2003. 34(9): P. 924-8.
13. MELK, A., ET AL., EXPRESSION OF P16INK4A AND OTHER CELL CYCLE REGULATOR AND SENESCENCE ASSOCIATED GENES IN AGING HUMAN KIDNEY. KIDNEY INT, 2004. 65(2): P. 510-20.
14. MELK, A., ET AL., EFFECTS OF DONOR AGE AND CELL SENESCENCE ON KIDNEY ALLOGRAFT SURVIVAL. AM J TRANSPLANT, 2009. 9(1): P. 114-23.
15. HARMAN, D., AGING: A THEORY BASED ON FREE RADICAL AND RADIATION CHEMISTRY. J GERONTOL, 1956. 11(3): P. 298-300.
16. OJO, A.O., ET AL., SURVIVAL IN RECIPIENTS OF MARGINAL CADAVERIC DONOR KIDNEYS COMPARED WITH OTHER RECIPIENTS AND WAIT-LISTED TRANSPLANT CANDIDATES. J AM SOC NEPHROL, 2001. 12(3): P. 589-97.
17. MERION, R.M., ET AL., DECEASED-DONOR CHARACTERISTICS AND THE SURVIVAL BENEFIT OF KIDNEY TRANSPLANTATION. JAMA, 2005. 294(21): P. 2726-33.
18. TUTTLE-NEWHALL, J.E., ET AL., ORGAN DONATION AND UTILIZATION IN THE UNITED STATES: 1998-2007. AM J TRANSPLANT, 2009. 9(4 PT 2): P. 879-93.
19. PASUPATHY, S. AND S. HOMER-VANNIASINKAM, SURGICAL IMPLICATIONS OF ISCHEMIC PRECONDITIONING. ARCH SURG, 2005. 140(4): P. 405-9; DISCUSSION 410.

Chapter 2

Mechanisms of ageing in chronic allograft nephropathy

D Susa, RWF de Bruin, JR Mitchell*, HP Roest, JHJ Hoeijmakers*, JNM IJzermans

Departments of Surgery, and Cell Biology and Genetics*, Erasmus Medical Center, Rotterdam, The Netherlands

Journal of Organ Dysfunction 2007; 3 (1): 7-17

ABSTRACT

Chronic allograft nephropathy (CAN) is the single most important cause of late graft loss. Histologic signs of CAN overlap with the histology of normally ageing kidneys. Clinical observations show an inverse relationship between either donor age or ischemia time and long-term graft survival. The mechanisms by which donor age or ischemia lead to reduced functional lifespan are currently unknown.

A review of the current literature on renal transplantation, ageing mechanisms and advances in the molecular pathogenesis of CAN was performed.

Ischemia time and donor age have been identified as major allo-antigen-independent risk factors. Following transplantation, reactive oxygen species cause oxidative DNA and telomeric damage. Evidence for a molecular connection between oxidative DNA damage and ageing has increased, and suggests that the mechanisms causing normal ageing and long-term organ dysfunction may be fundamentally the same. The accelerated appearance of ageing phenotypes in transplanted organs has been shown in several studies. Together with the deleterious ongoing allogeneic immune response of the recipient, this leads to accelerated exhaustion of the regenerative capacity of the kidney, and eventually to dysfunction of the renal allograft.

INTRODUCTION

Due to refinements in tissue typing, advancements in organ preservation, surgical techniques and more effective immunosuppressive agents, the short-term results after clinical organ transplantation have improved progressively. For example, one year survival of cadaveric kidneys had increased from approximately 40% by the end of the 1960s to about 89% [1] today, and for living-donor kidneys from 80 to 95% [1, 2]. Despite these promising short-term results, the rate of late graft failure, defined as greater than 1 year after transplantation, has not seriously improved for decades. For instance, the half-life of cadaver kidney allografts has remained consistently at 7.5-8.8 years [3, 4]. Chronic Allograft Nephropathy (CAN) is the most important single cause of late graft deterioration and failure. Clearly, there is a need for better understanding of the etiology of this disease and for the development of effective strategies to prevent or halt this chronic attrition of transplanted organs.

CHRONIC ALLOGRAFT NEPHROPATHY

CAN is a phenomenon in renal allografts characterized by a gradual deterioration in graft function months to years after transplantation, eventually leading to graft failure, accompanied by characteristic morphologic features. Clinically, CAN manifests itself as a slowly progressive decline in glomerular filtration rate and is often associated with proteinuria and arterial hypertension, usually occurring after 90 days post-transplantation [5-7]. Histologic markers of CAN include intimal hyperplasia of graft arteries, glomerular sclerosis, tubular atrophy and interstitial fibrosis [7-9]. These histologic features have been found in protocol biopsies 2 years post-transplantation from as much as 67% of recipients of cadaveric kidney transplants [10, 11].

RISK FACTORS AND MECHANISMS OF CAN

Until now the exact mechanisms of CAN are poorly understood. Several allo-antigen dependent and allo-antigen independent risk factors (Table 1) have been proposed to be involved in the pathophysiology leading to CAN [8].

Table 1. Risk factors for chronic allograft nephropathy

Allo-antigen dependent factors	Allo-antigen independent factors
Histoincompatibility	Ischemia reperfusion injury
Acute rejection	Brain death
Subclinical rejection	Infection
Calcineurin nephrotoxicity	Hyperlipedemia
Suboptimal immunosuppression	Age
Immunosuppressive agent	Gender
Anti-donor antibodies	Race
	Size

Risk factors for chronic allograft nephropathy

Allo-antigen dependent factors

Allo-antigen dependent risk factors, including histoincompatibility, acute rejection, subclinical rejection, suboptimal immunosuppression, CNI nephrotoxicity and different immunosuppressive agents, have been linked to CAN by clinical and experimental data. Long-term graft survival strongly correlates with the degree of histocompatibility between donor and recipient [12, 13].

Acute rejection episodes strongly correlate with the lifespan of a graft [14, 15]. Clinical studies have found that the onset, frequency and severity of an acute rejection episode are independent risk factors for CAN [16, 17]. While the impact on CAN of a single acute rejection episode with a complete functional recovery in the early posttransplant period is minimal, the more severe and later acute rejection episodes are associated with a higher chance to develop CAN. These clinical observations have been confirmed in several experimental studies [18-20].

Subclinical rejection (SCR), defined as the presence of histologic rejection without concurrent renal dysfunction [21], appears to precede CAN in serial protocol biopsies, suggesting a correlation between SCR and poorer long-term outcome [22, 23]. When evaluating the predictive value of SCR on long-term graft survival contradictory results have been found [24-27]. However, in a randomized prospective study, Rush et al [28, 29] showed improved long-term graft survival after treatment of SCR found in protocol biopsies with high dose steroids. Untreated SCR inflicts permanent tubulointerstitial damage and fibrosis [21, 30].

Since its introduction in organ transplantation in the 1980s, calcineurin inhibitors (CNI) have been the cornerstone of immunosuppressive therapy in renal transplant recipients, owing it to a dramatic reduction in the incidence and severity of acute rejection [31-34]. Although short-term results improved significantly, long term patient and graft survival have not improved in a similar manner. This can partly be attributed to the nephrotoxic side-effects of CNIs [35-37]. CNI toxicity is classified in two phases: an acute, functional, dose-dependent decrease in renal blood flow and glomerular filtration rate (GFR), and a chronic, dose-independent form with structural changes (arteriolosclerosis, glomerulosclerosis, tubular atrophy, and interstitial fibrosis) leading to a progressive and persistent decrease in GFR [38]. Both clinical and experimental studies show that a low dose of cyclosporine is associated with CAN, while higher dosing or triple versus double dosing can reduce the incidence of CAN [39-41]. However, maintaining high doses of CNIs for a prolonged period is not recommended because of its toxic side effects.

Finally, the type of immunosuppressive agent can also play a role. For example, at 1-year post-transplantation the incidence and histologic severity of acute rejection is significantly lower in tacrolimus treated patients compared to cyclosporine treated ones. Subsequently, 3- and 5-years post-transplantation, a decreased risk of allograft failure was seen in patients receiving tacrolimus compared to patients receiving cyclosporine [42, 43].

Allo-antigen independent factors

CAN can also develop in human kidney transplants between identical twins in the absence of an allo-immune response. Several allo-antigen independent factors have been identified, including ischemia reperfusion injury, brain death and donor age.

Ischemia reperfusion injury is thought to be a critical risk factor for both early delayed graft function (DGF) and late allograft dysfunction. In clinical transplantation it has been shown that prolonged ischemia is a strong risk factor for DGF and long-term graft loss [44-47]. In particular, Salahudeen and colleagues demonstrated that prolonged ischemia time is a significant predictor of long-term graft survival [44]. In this study ischemia time was analyzed as an univariate among 6465 patients who received a kidney-only transplant of cadaveric origin for the first time in 1995. Ischemia time had a significant effect on the 6-year allograft survival; a 10 hour increase in ischemia time was associated with a hazard risk ratio of 1.20 for graft failure that persisted after adjusting for donor age, recipient age and race, HLA mismatch and rejection treatments in the first 6 months. Six year graft survival was progressively worse for the categories with ischemia times between 0 and 10 hours, 11 to 20 hours, 21 to 30 hours respectively and significantly worse for ischemia time >30 hours. Likewise, Vistoli et al showed increased DGF and reduced 3-year graft survival with ischemia time longer than 16 hours in kidneys from donors older than 65 years [45]. Experimental studies have confirmed that ischemia reperfusion injury can also cause CAN-like lesions in the absence of allogenicity [48, 49]. Previous work from our laboratory demonstrated that rat kidney isografts with 24 hours cold ischemia develop the same functional and morphologic changes as seen in allografts. Similarly, Tilney showed that clamping of the renal pedicle of an uninephrectomized rat leads to the same functional changes as seen in isografts at 1 year post-surgery [50]. These changes appear to be induced by the oxidative stress of ischemia reperfusion injury.
Brain death resulting from trauma or intracranial hemorrhage is associated with a variety of adverse effects on donor organs prior to transplantation. The hypothesis has been put forward that brain death activates surface molecules on peripheral organs via cytokines; in brain death donors, increased serum cytokine levels, such as IL-1β, IL-6, IL-8, TNF-α, and RANTES are found before organ procurement [51]. Experimental models of brain death demonstrate increased activation of endothelial cells [52, 53] and the tempo of acute rejection is increased in organs from brain dead animals [54, 55]. In addition, the rise of intracranial pressure due to cerebral edema results in cerebral herniation. This triggers a massive release of catecholamines, causing profound vasoconstriction and endothelial injury [56]. Endothelial activation coupled with release of cytokines, complement activation and depletion of tissue plasminogen activator leads to a procoagulant state [57]. Other aberrations include the initial release of anterior pituitary hormones, which leads to a subsequent reduction in the levels of circulating thyroid hormone, cortisol, insulin and antidiuretic hormone [58]. Diabetes insipidus rapidly occurs, and cardiac arrhythmias and rapid fluctuations in blood pressure are common. All these factors may obviously adversely affect the function and integrity of the allograft.
The increasing gap between supply and demand of donor kidneys has led to an increase in the use of donors older than 50 years. Several clinical studies have demonstrated that increased donor age is associated with increased short- and long-term allograft dysfunction [59, 60]. The effect of donor age on short-term graft survival in 30,000 transplantations, performed between 1987 and 1991, was also described by Alexander and co-workers [61]: with each decade of increase in

donor age, the relative risk of graft failure at 1 year rose by 15-20%. However, the magnitude of this effect increased exponentially with time. Graft failure at 2 years reached an odds ratio of 3.25 in donors older than 70 years, as compared to a 30 year old donor group. In 1996, Gjertson [62] reported the long-term graft outcome with regard to donor age. In kidney allografts surviving the first post-operative year, donor age accounted for 30% of the variability in 5-year outcome. This decreased survival may explained by the observation that old kidneys have a lower nephron mass, more intersititial fibrosis and likely display a decreased intrinsic capacity to recover from injury [63].

Mechanisms of CAN

With modern immunosuppressive agents, excellent long-term clinical results obtained from living-related but also unrelated (random HLA match) donors have further supported the importance of alloimmune-independent mechanisms of graft injury [8, 64]. Currently, non-alloimmune specific risk factors related to the donor and the graft are associated with the development of CAN. These include ischemia time and donor age [47, 60, 65].

Ischemia-induced damage becomes apparent during the reperfusion of the transplanted organ. Reactive oxygen species (ROS) are formed, which damage vascular endothelial and other cells by causing oxidative DNA damage and lipid peroxidation in the cell membrane. This is thought to initiate the poorly understood pathogenetic process leading to late graft loss. Oxidative damage induces parenchymal injury and may initially kill a large number of cells, forcing proliferation to replenish the lost cells (Figure 1). Expression of adhesion molecules facilitates infiltration of leukocytes in the kidney, which causes more tissue injury and leads to an innate immune response. Damaged graft cells also facilitate immune recognition via their own MHC molecules and thus promote the adaptive immune response (rejection), leading to more tissue injury and cell death. This may give rise to a self-sustaining mechanism with cycles of cell death and proliferation that eventually trigger cellular senescence, exhaustion of the proliferative potential and organ dysfunction. Donor age further enhances this phenomenon. Virtually all studies indicate that older donor age has an adverse effect on graft survival. Most studies find that older kidneys are more likely to undergo acute rejection. Reasons for this are currently unknown, but it has been suggested that older kidneys are more immunogenic [66]. Recent evidence has shown that basal serum IL-6 levels increase with age, and ageing induces a pro-inflammatory state, which may well explain the higher immunogenicity of older kidneys [67].

In 1999 Phillip Halloran introduced the concept of accelerated senescence in chronic allograft nephropathy, proposing that ageing, nonimmune injury and rejection drive renal tissue down a common pathway to a state of depletion of finite repair potential [63]. In addition, several recent studies have shown accelerated appearance of ageing phenotypes in transplanted organs, which suggest that mechanisms of ageing are involved in the development of chronic renal allograft nephropathy. Next, we will discuss mechanisms of ageing and how these can be fitted in the concept of CAN.

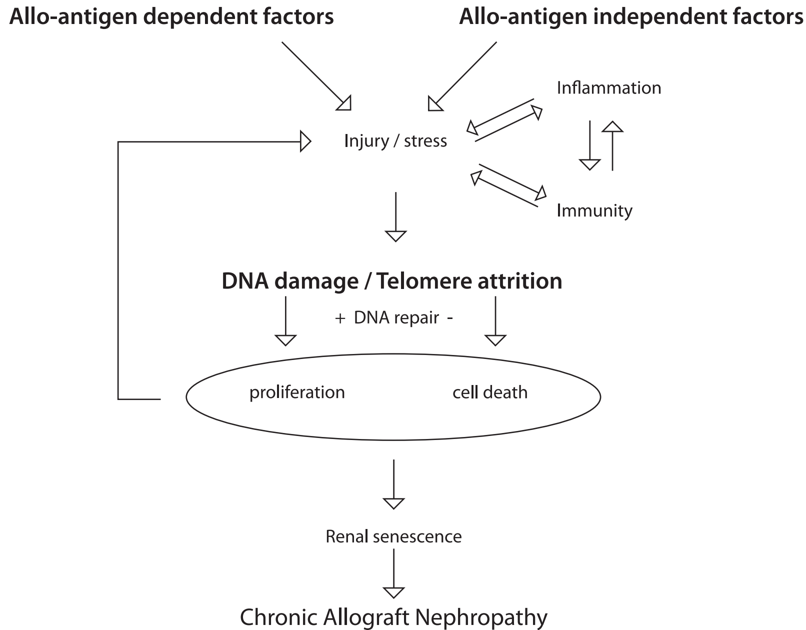

Figure 1. Allo-antigen-dependent and -independent factors contribute to ongoing injury renal cells during transplantation

MECHANISMS OF AGEING

Ageing is a complex process that is poorly understood at either the cellular or molecular level. The main cause of ageing is thought to be by the accumulation of somatic damage. Among the various types of somatic damage, oxidative damage by reactive oxygen species (ROS) is considered to be the leading cause of ageing [68]. ROS produced by normal cellular metabolism are a primary source of DNA damage. More than 100 different types of oxidative modifications have been identified in DNA [69]. These vary from a wide diversity of base and sugar modifications, to different types of single- and double-strand DNA breaks and to alterations in telomeres [70] (Figure 2). Disruption of vital processes such as transcription and replication by these modifications may lead to impaired cellular function, cell death or terminal growth arrest. ROS are also thought to be the source of oxidative damage sustained by organs during reperfusion after transplantation [71, 72].

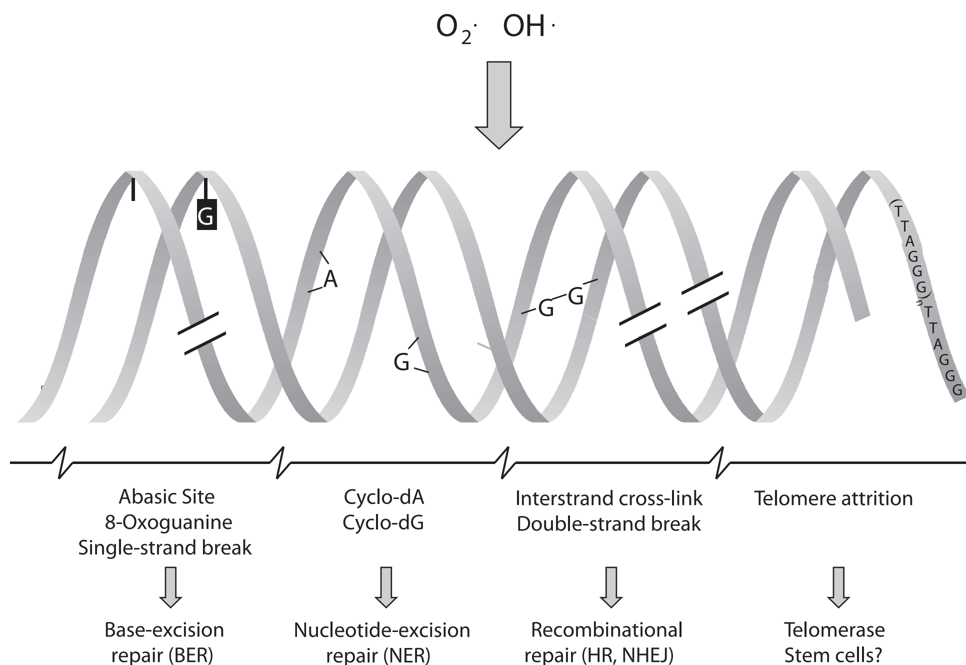

Figure 2. ROS as illustrated by O2. and OH., are a primary source of cellular damage. Adapted from Hoeijmakers [72].

DNA damage and repair

To counteract oxidative DNA damage, nature has evolved several efficient, highly conserved DNA repair mechanisms, with partially overlapping substrate specificity [73]. These repair pathways can be roughly divided as follows: two excision repair pathways deal with modifications affecting only one of the DNA strands. The lesion is taken out in a "cut and patch" type manner, and the gap is filled in, using the intact complementary strand as a template. Nucleotide excision repair (NER) deals primarily with helix-distorting base modifications, while base excision repair (BER) removes small lesions caused by oxidative damage. Lesions on the transcribed DNA strand that block elongating RNA polymerases are targeted by transcription-coupled repair (TCR) in a process overlapping largely with NER, while lesions that block replicative DNA polymerases or by other recombination-based bypass mechanisms in favor of repair at a later time. These processes are critical for coping with ROS-induced base alterations and single-strand breaks. One of the most severe types of DNA lesions induced indirectly by ROS is a double strand break. Double-strand breaks (DSB)s are repaired by homologous recombination (HR) or the less accurate nonhomologous end joining (NHEJ), depending on the availability of an intact DNA copy or not, respectively. Deficiencies in any of these repair mechanism give rise to several human heritable syndromes [74, 75]. These disorders are either characterized by a high predisposition to cancer due to increased damage-induced mutation frequency and/or exhibit accelerated aging features, caused by enhanced damage-triggered cell death or cellular senescence. The latter conditions are termed seg-

mental progeriod syndromes, because they all display early onset of some but not all features associated with normal ageing. At least three human disorders are associated with heritable defects in NER: xeroderma pigmentosum (XP), trichothiodystrophy (TTD) and Cockayne syndrome (CS) [76]. Mouse models of these diseases display similar features of premature ageing like their human counterparts [77, 78]. In contrast hereditary defects in core BER components have not yet been identified in humans, most likely because they are lethal at the embryonic stage of development: in mice, inactivation of genes essential for core BER reaction generally causes embryonic mortality [79]. Several rare human syndromes are also associated with defects in the repair or proper response to DSB, including ataxia telangiectasia (AT), Bloom syndrome and Werner syndrome (WS) [80, 81].
Thus, DNA damage caused by intrinsic and extrinsic oxidative stress can lead to a premature ageing phenotype, if not properly maintained by this intricate network of DNA repair pathways [82, 83], lending strong support to time-dependent accumulation of oxidative DNA damage as a primary root cause of organismal ageing.

Telomeres

Telomeres are the physical ends of linear chromosomes, consisting of a variable number of tandem $(TTAGGG)_n$ repeats of DNA [84]. With each cell division they shorten, and when the shortening reaches a critical limit, chromatin starts to alter, leading to a halt in cell cycling by activation of a damage-sensing checkpoint or forcing cells to undergo DNA repair rearrangements [85]. In such a situation, cells cease to divide and reach a quiescent state termed replicative senescence. This situation can be prevented by the ribonucleoprotein enzyme telomerase, a specialized reverse transcriptase enzyme that can add telomeric repeats de novo to the 5' end of telomeres based on the template provided by its integral RNA component (Terc). Active telomerase copies a region within its integral RNA component enabling chromosome 3' ends extension by repeats of telemeric sequence [86]. Embryonic stem cells exhibit unlimited self-renewal capacity due to the expression of telomerase [87], while most human somatic cells exhibit low or absent telomerase activity beyond the early stages of development [88]. These telomerase negative cells experience progressive telomere attrition with each mitotic cycle, both in cell culture [89] and during ageing of the whole organism [90, 91]. Constitutive expression of human telomerase catalytic subunit (hTERT) can extend the lifespan in numerous cell types [92], while accelerated telomere shortening is seen in several human premature ageing syndromes (e.g. Werner syndrome, ataxia telangiectasia, dyskeratosis congenita) [93-95], arguing for a central role of telomere maintenance in at least some aspects of normal ageing. Telomere length in a given cell thus may serve as a marker of its replicative history and of the residual capacity for further cell division both in highly proliferative tissues such as skin, gut and blood and perhaps as well in regeneration of organs including liver and kidney that are not normally highly proliferative but whose cells have an intrinsic capacity to proliferate upon damage.

Cellular senescence

By the classic experiment of Hayflick and Moorehead cellular senescence has been defined as an irreversible loss of replicative capacity, occurring in primary human fibroblasts after a reproducible number of population doublings [96]. This number, termed the Hayflick limit, is fairly constant under standard cell culture conditions, but can be modified substantially by increasing or decreasing, especially oxidative stress levels [97]. There is a general correlation between donor age and Hayflick limit, with a higher fraction of cells displaying markers of a senescent phenotype in tissues from older donors and shorter Hayflick limits for cultures from donors with premature ageing syndromes [98, 99]. Senescent cells remain alive and metabolically active for many months but present atypical features when compared to their replication competent counterparts, including altered morphology (increased cell surface and volume, decreased cell saturation density), lipofuscin accumulation, changes in the activity of several enzymes [100-102], including increased activity of acidic ß-galactosidase [103], changes in the expression of many genes at the mRNA and protein levels [104-106] and decreased telomere length [107, 108]. Senescent fibroblasts produce large amounts of ROS like H_2O_2. They contain 30% more 8-oxo-dG in DNA and four times as many free 8-oxo-Guanidine bases, pointing to the accumulation of oxidative DNA damage [109].
The numerical reproducibility of the Hayflick limit immediately suggested the idea of a 'biological clock' or, more specifically, a replication counter that counts biological time in numbers of cell divisions and after a reproducible number of divisions would trigger signaling pathways that block cellular growth [110]. Several processes have been put forward as possible counting mechanisms, of which telomere attrition is the most established one [111]. Recent work indicates that it is not the length of the telomeres themselves that triggers a senescent arrest but rather the stability of the telomeric loop. Telomere shortening is thought to destabilize the telomeric loop, thereby increasing the probability of uncapping the telomeres [112, 113].
Cellular senescence is not only triggered by the telomere driven checkpoint, but also by several other pathways. For instance, overexpression of certain oncogenes induces a senescent phenotype in primary mouse and human cells [114, 115]. In human epithelial cells, suboptimal culture conditions can trigger a telomere-independent, p16 dependent growth arrest [116]. Inhibitors of histone deacetylases modify chromatin towards a more decondensed structure, thereby inducing a senescent-like growth arrest [117]. Finally, DNA-damaging stress can induce cellular senescence. Several types of stress have been demonstrated, including drugs generating double strand breaks [118], different kinds of radiation [119, 120] and different means of generating chronic and acute oxidative stress [121-123]. Oxidative stress can modify the rate of telomere shortening [124, 125]. Telomeres accumulate single and double strand breaks under oxidative stress. During replication polymerases are stalled transiently. This might lead to premature termination of replication at the telomeres and to larger attrition of telomeres than would be the case by the end replication problem alone [126]. Likewise, an inverse correlation was found between antioxidant defence capacity and the rate of telomere shortening [127], suggesting that ROS-mediated DNA damage is a major cause of cellular senescence.
Mechanistically and phenotypically, telomere-driven and stress-induced premature senescence

are very similar [128-130]. It appears that similar phenotypes are triggered either by a pre-programmed cell division counter or by random DNA stress and damage and inability to repair this damage. Thus, cellular senescence is to a large extent governed by internally and externally generated (oxidative) stress and the cells ability to cope with it.

MECHANISMS OF AGEING IN RENAL SENESCENCE

Deterioration of renal function during normal ageing is among the most dramatic of any organ or organ system, and are both functional and structural [131]. The number of functioning glomeruli declines roughly in accord with the changes in renal weight, while the size of the remaining glomeruli increases [132, 133]. This results in a decline in glomerular filtration rate (GFR), a rise in renal vascular resistance, decline in renal blood flow and an increase in basement membrane permeability, leading to an increase in urinary protein excretion [134]. The structural phenotype of renal senescence is associated with both total and cortical renal mass decrease with age [135, 136]. Other histological features are hyalinosis and fibrous thickening of the arterial intima, interstitial fibrosis, global glomerular sclerosis, focal tubular atrophy, and patchy inflammation [137].

The cellular and molecular pathways of renal ageing are still poorly understood. Recently the mechanisms involved in cellular senescence (in vitro) have also been implicated to play a role in normal ageing kidneys [138, 139]. Melk and colleagues have demonstrated that aged human kidneys show features of cellular senescence similar to cultured somatic cells. With age, an increase in the expression of the cyclin-dependent kinase inhibitor $p16^{INK4a}$ in renal cortex was seen. Also, increased $p16^{INK4a}$ and p53 levels, markers and mediators in cellular senescence in cultured human and mouse cells [140-143], correlated with the degree of glomerulosclerosis, interstitial fibrosis and tubular atrophy. Likewise, telomere shortening has been described in ageing kidneys [144]. Experimental studies show similar results; cellular senescence markers, including $p16^{INK4a}$, lipofuscin and senescence-associated ß-galactosidase (SA-ß-Gal) increase and telomere length decreases in rat kidney with growth and age [145, 146]. These clinical and experimental senescence associated changes have also been shown in other organs and organ systems, including liver, lung, heart, peripheral blood cells and vascular tissue [147-151]. When these organs or organ systems are subjected to intrinsic and extrinsic environmental stresses, like chronic disease or increased oxidative stress around transplantation, the burden of proliferative demand is increased which may lead to premature senescence with increased expression of several cellular senescence markers. Cellular senescence has been reported to play a important role in the progression of cardiovascular disease [152-154], chronic hepatitis and liver cirrhosis [155-157], and bone marrow transplantation [158, 159].

In case of transplanted kidneys, CAN is the major cause of long-term transplant loss other than patient death [8]. Kidneys suffering from CAN have histologic features resembling normal aged kidneys, like intimal hyperplasia of graft arteries, glomerular sclerosis, tubular atrophy and interstitial fibrosis [7-9, 63, 160] and show similar gradual deterioration in function only at an accelerated pace in months to years after transplantation [5-7]. In recent years both clinical and experimental studies show that, when kidneys are faced with excessive stress associated with transplantation,

cellular senescence is accelerated. It has been shown that the senescence biomarker SA-ß-Gal is expressed in 67% in kidney biopsies of chronic allograft nephropathy, compared to 15 % in control biopsy specimens [161]. SA-ß-Gal positive cells had shorter telomeres than cells that did not express the marker. A clear correlation is demonstrated between the severity of CAN and the percentage of senescent cells. Also shown is the correlation between the presence of senescent tubular epithelial cells and donor age, one of the risk factors of CAN summed up in Table 1. Studies indicate an increased expression of cyclin-dependent kinase inhibitors $p16^{INK4a}$, $p27^{Kip1}$ [162] and $p21^{WAF1/CIP1}$ [163] in CAN. $p21^{WAF1/CIP1}$ expression in kidneys with CAN correlates with the number of acute rejection episodes. Cyclin-dependent kinase inhibitors play an important role in inducing the senescent phenotype [164, 165]. Oxidative stress has been implicated in accelerated telomere attrition [110] and recent experimental work suggests that (oxidative stress in the form of) ischemia reperfusion injury contributes to telomere attrition in a kidney transplantation model [166]. Thus, oxidative stress, sustained during transplantation, causes DNA and telomeric damage and, unless efficiently repaired, contributes to renal senescence. Together with the deleterious effects of brain death, donor age, nephrotoxic immunosuppression and an ongoing allogeneic immune reponse of the recipient, this leads to exhaustion of the regenerative capacity of the kidney over time, and eventually to dysfunction of the renal allograft.

Insights in the mechanisms of oxidative DNA stress and subsequent repair may significantly contribute to our understanding of the etiology of CAN, which may lead to additional preventive or therapeutic measures to prolong kidney graft survival.

REFERENCES

1. CECKA, J.M., THE UNOS RENAL TRANSPLANT REGISTRY. CLIN TRANSPL, 2002: P. 1-20.
2. TERASAKI, P.I., ET AL., THIRTY-YEAR TRENDS IN CLINICAL KIDNEY TRANSPLANTATION. CLIN TRANSPL, 1993: P. 553-62.
3. GJERTSON, D.W., SURVIVAL TRENDS IN LONG-TERM FIRST CADAVER-DONOR KIDNEY TRANSPLANTS. CLIN TRANSPL, 1991: P. 225-35.
4. MEIER-KRIESCHE, H.U., J.D. SCHOLD, AND B. KAPLAN, LONG-TERM RENAL ALLOGRAFT SURVIVAL: HAVE WE MADE SIGNIFICANT PROGRESS OR IS IT TIME TO RETHINK OUR ANALYTIC AND THERAPEUTIC STRATEGIES? AM J TRANSPLANT, 2004. 4(8): P. 1289-95.
5. PAUL, L.C., CHRONIC REJECTION OF ORGAN ALLOGRAFTS: MAGNITUDE OF THE PROBLEM. TRANSPLANT PROC, 1993. 25(2): P. 2024-5.
6. VAZQUEZ, M.A., CHRONIC REJECTION OF RENAL TRANSPLANTS: NEW CLINICAL INSIGHTS. AM J MED SCI, 2000. 320(1): P. 43-58.
7. MONACO, A.P., ET AL., CURRENT THINKING ON CHRONIC RENAL ALLOGRAFT REJECTION: ISSUES, CONCERNS, AND RECOMMENDATIONS FROM A 1997 ROUNDTABLE DISCUSSION. AM J KIDNEY DIS, 1999. 33(1): P. 150-60.
8. KOUWENHOVEN, E.A., J.N. IJZERMANS, AND R.W. DE BRUIN, ETIOLOGY AND PATHOPHYSIOLOGY OF CHRONIC TRANSPLANT DYSFUNCTION. TRANSPL INT, 2000. 13(6): P. 385-401.
9. SOLEZ, K., ET AL., INTERNATIONAL STANDARDIZATION OF CRITERIA FOR THE HISTOLOGIC DIAGNOSIS OF RENAL ALLOGRAFT REJECTION: THE BANFF WORKING CLASSIFICATION OF KIDNEY TRANSPLANT PATHOLOGY. KIDNEY INT, 1993. 44(2): P. 411-22.
10. SOLEZ, K., F. VINCENTI, AND R.S. FILO, HISTOPATHOLOGIC FINDINGS FROM 2-YEAR PROTOCOL BIOPSIES FROM A U.S. MULTICENTER KIDNEY TRANSPLANT TRIAL COMPARING TAROLIMUS VERSUS CYCLOSPORINE: A REPORT OF THE FK506 KIDNEY TRANSPLANT STUDY GROUP. TRANSPLANTATION, 1998. 66(12): P. 1736-40.
11. WOMER, K.L., J.P. VELLA, AND M.H. SAYEGH, CHRONIC ALLOGRAFT DYSFUNCTION: MECHANISMS AND NEW APPROACHES TO THERAPY. SEMIN NEPHROL, 2000. 20(2): P. 126-47.
12. TAKEMOTO, S.K., ET AL., TWELVE YEARS' EXPERIENCE WITH NATIONAL SHARING OF HLA-MATCHED CADAVERIC KIDNEYS FOR TRANSPLANTATION. N ENGL J MED, 2000. 343(15): P. 1078-84.
13. SMITH, J.D., ET AL., REDUCTION OF CELLULAR REJECTION AND INCREASE IN LONGER-TERM SURVIVAL AFTER HEART TRANSPLANTATION AFTER HLA-DR MATCHING. LANCET, 1995. 346(8986): P. 1318-22.
14. HARIHARAN, S., ET AL., IMPROVED GRAFT SURVIVAL AFTER RENAL TRANSPLANTATION IN THE UNITED STATES, 1988 TO 1996. N ENGL J MED, 2000. 342(9): P. 605-12.
15. GJERTSON, D.W., DETERMINANTS OF LONG-TERM SURVIVAL OF ADULT KIDNEY TRANSPLANTS: A 1999 UNOS UPDATE. CLIN TRANSPL, 1999: P. 341-52.
16. MASSY, Z.A., ET AL., CHRONIC RENAL ALLOGRAFT REJECTION: IMMUNOLOGIC AND NONIMMUNOLOGIC RISK FACTORS. KIDNEY INT, 1996. 49(2): P. 518-24.
17. COSIO, F.G., ET AL., RENAL ALLOGRAFT SURVIVAL FOLLOWING ACUTE REJECTION CORRELATES WITH BLOOD PRESSURE LEVELS AND HISTOPATHOLOGY. KIDNEY INT, 1999. 56(5): P. 1912-9.
18. YILMAZ, S., ET AL., CHRONIC RENAL ALLOGRAFT REJECTION CAN BE PREDICTED BY THE SURFACE AREA UNDER THE SERUM CREATININE VERSUS TIME CURVE. TRANSPLANT PROC, 1995. 27(1): P. 927.
19. NAKAGAWA, T., ET AL., ACUTE REJECTION ACCELERATES GRAFT CORONARY DISEASE IN TRANSPLANTED RABBIT HEARTS. CIRCULATION, 1995. 92(4): P. 987-93.
20. HIRT, S.W., ET AL., THE INFLUENCE OF ISCHEMIA ON THE SEVERITY OF CHRONIC REJECTION IN A RAT LUNG TRANSPLANT MODEL. TRANSPLANT PROC, 1999. 31(1-2): P. 189-90.
21. RUSH, D., ET AL., EFFECT OF SUBCLINICAL REJECTION ON RENAL ALLOGRAFT HISTOLOGY AND FUNCTION AT 6 MONTHS. TRANSPLANT PROC, 1996. 28(1): P. 494-5.
22. NANKIVELL, B.J., ET AL., THE NATURAL HISTORY OF CHRONIC ALLOGRAFT NEPHROPATHY. N ENGL J MED, 2003. 349(24): P. 2326-33.
23. SHISHIDO, S., ET AL., THE IMPACT OF REPEATED SUBCLINICAL ACUTE REJECTION ON THE PROGRESSION OF CHRONIC ALLOGRAFT NEPHROPATHY. J AM SOC NEPHROL, 2003. 14(4): P. 1046-52.
24. MORESO, F., ET AL., SUBCLINICAL REJECTION ASSOCIATED WITH CHRONIC ALLOGRAFT NEPHROPATHY IN PROTOCOL BIOPSIES AS A RISK FACTOR FOR LATE GRAFT LOSS. AM J TRANSPLANT, 2006. 6(4): P. 747-52.
25. ROBERTS, I.S., ET AL., SUBCLINICAL REJECTION AND BORDERLINE CHANGES IN EARLY PROTOCOL BIOPSY SPECIMENS AFTER RENAL TRANSPLANTATION. TRANSPLANTATION, 2004. 77(8): P. 1194-8.

26. SERON, D., ET AL., PROTOCOL RENAL ALLOGRAFT BIOPSIES AND THE DESIGN OF CLINICAL TRIALS AIMED TO PREVENT OR TREAT CHRONIC ALLOGRAFT NEPHROPATHY. TRANSPLANTATION, 2000. 69(9): P. 1849-55.
27. CHOI, B.S., ET AL., CLINICAL SIGNIFICANCE OF AN EARLY PROTOCOL BIOPSY IN LIVING-DONOR RENAL TRANSPLANTATION: TEN-YEAR EXPERIENCE AT A SINGLE CENTER. AM J TRANSPLANT, 2005. 5(6): P. 1354-60.
28. RUSH, D., ET AL., BENEFICIAL EFFECTS OF TREATMENT OF EARLY SUBCLINICAL REJECTION: A RANDOMIZED STUDY. J AM SOC NEPHROL, 1998. 9(11): P. 2129-34.
29. RUSH, D.N., ET AL., PROTOCOL BIOPSIES IN RENAL TRANSPLANTATION: RESEARCH TOOL OR CLINICALLY USEFUL? CURR OPIN NEPHROL HYPERTENS, 1998. 7(6): P. 691-4.
30. NANKIVELL, B.J., ET AL., DELTA ANALYSIS OF POSTTRANSPLANTATION TUBULOINTERSTITIAL DAMAGE. TRANSPLANTATION, 2004. 78(3): P. 434-41.
31. CYCLOSPORIN IN CADAVERIC RENAL TRANSPLANTATION: ONE-YEAR FOLLOW-UP OF A MULTICENTRE TRIAL. LANCET, 1983. 2(8357): P. 986-9.
32. A RANDOMIZED CLINICAL TRIAL OF CYCLOSPORINE IN CADAVERIC RENAL TRANSPLANTATION. N ENGL J MED, 1983. 309(14): P. 809-15.
33. VINCENTI, F., ET AL., ONE-YEAR FOLLOW-UP OF AN OPEN-LABEL TRIAL OF FK506 FOR PRIMARY KIDNEY TRANSPLANTATION. A REPORT OF THE U.S. MULTICENTER FK506 KIDNEY TRANSPLANT GROUP. TRANSPLANTATION, 1996. 61(11): P. 1576-81.
34. MAYER, A.D., ET AL., MULTICENTER RANDOMIZED TRIAL COMPARING TACROLIMUS (FK506) AND CYCLOSPORINE IN THE PREVENTION OF RENAL ALLOGRAFT REJECTION: A REPORT OF THE EUROPEAN TACROLIMUS MULTICENTER RENAL STUDY GROUP. TRANSPLANTATION, 1997. 64(3): P. 436-43.
35. CAMPISTOL, J.M. AND S.H. SACKS, MECHANISMS OF NEPHROTOXICITY. TRANSPLANTATION, 2000. 69(12 SUPPL): P. SS5-10.
36. VERCAUTEREN, S.B., ET AL., A META-ANALYSIS AND MORPHOLOGICAL REVIEW OF CYCLOSPORINE-INDUCED NEPHROTOXICITY IN AUTO-IMMUNE DISEASES. KIDNEY INT, 1998. 54(2): P. 536-45.
37. OJO, A.O., ET AL., CHRONIC RENAL FAILURE AFTER TRANSPLANTATION OF A NONRENAL ORGAN. N ENGL J MED, 2003. 349(10): P. 931-40.
38. BENNETT, W.M., THE NEPHROTOXICITY OF NEW AND OLD IMMUNOSUPPRESSIVE DRUGS. REN FAIL, 1998. 20(5): P. 687-90.
39. ALMOND, P.S., ET AL., RISK FACTORS FOR CHRONIC REJECTION IN RENAL ALLOGRAFT RECIPIENTS. TRANSPLANTATION, 1993. 55(4): P. 752-6; DISCUSSION 756-7.
40. ISONIEMI, H.M., ET AL., HISTOPATHOLOGICAL FINDINGS IN WELL-FUNCTIONING, LONG-TERM RENAL ALLOGRAFTS. KIDNEY INT, 1992. 41(1): P. 155-60.
41. GEERLING, R.A., ET AL., SUPPRESSION OF ACUTE REJECTION PREVENTS GRAFT ARTERIOSCLEROSIS AFTER ALLOGENEIC AORTA TRANSPLANTATION IN THE RAT. TRANSPLANTATION, 1994. 58(11): P. 1258-63.
42. JENSIK, S.C., TACROLIMUS (FK 506) IN KIDNEY TRANSPLANTATION: THREE-YEAR SURVIVAL RESULTS OF THE US MULTICENTER, RANDOMIZED, COMPARATIVE TRIAL. FK 506 KIDNEY TRANSPLANT STUDY GROUP. TRANSPLANT PROC, 1998. 30(4): P. 1216-8.
43. VINCENTI, F., ET AL., A LONG-TERM COMPARISON OF TACROLIMUS (FK506) AND CYCLOSPORINE IN KIDNEY TRANSPLANTATION: EVIDENCE FOR IMPROVED ALLOGRAFT SURVIVAL AT FIVE YEARS. TRANSPLANTATION, 2002. 73(5): P. 775-82.
44. SALAHUDEEN, A.K., N. HAIDER, AND W. MAY, COLD ISCHEMIA AND THE REDUCED LONG-TERM SURVIVAL OF CADAVERIC RENAL ALLOGRAFTS. KIDNEY INT, 2004. 65(2): P. 713-8.
45. VISTOLI, F., ET AL., KIDNEY TRANSPLANTATION FROM DONORS AGED MORE THAN 65 YEARS. TRANSPLANT PROC, 2004. 36(3): P. 481-4.
46. HETZEL, G.R., ET AL., RISK FACTORS FOR DELAYED GRAFT FUNCTION AFTER RENAL TRANSPLANTATION AND THEIR SIGNIFICANCE FOR LONG-TERM CLINICAL OUTCOME. TRANSPL INT, 2002. 15(1): P. 10-6.
47. SHOSKES, D.A. AND J.M. CECKA, DELETERIOUS EFFECTS OF DELAYED GRAFT FUNCTION IN CADAVERIC RENAL TRANSPLANT RECIPIENTS INDEPENDENT OF ACUTE REJECTION. TRANSPLANTATION, 1998. 66(12): P. 1697-701.
48. KOUWENHOVEN, E.A., ET AL., LATE GRAFT DYSFUNCTION AFTER PROLONGED COLD ISCHEMIA OF THE DONOR KIDNEY: INHIBITION BY CYCLOSPORINE. TRANSPLANTATION, 1999. 68(7): P. 1004-10.
49. TULLIUS, S.G., ET AL., LONG-TERM KIDNEY ISOGRAFTS DEVELOP FUNCTIONAL AND MORPHOLOGIC CHANGES THAT MIMIC THOSE OF CHRONIC ALLOGRAFT REJECTION. ANN SURG, 1994. 220(4): P. 425-32; DISCUSSION 432-5.
50. AZUMA, H., ET AL., CELLULAR AND MOLECULAR PREDICTORS OF CHRONIC RENAL DYSFUNCTION AFTER INITIAL

ISCHEMIA/REPERFUSION INJURY OF A SINGLE KIDNEY. TRANSPLANTATION, 1997. 64(2): P. 190-7.
51. PRATSCHKE, J., ET AL., ACTIVATION OF PROINFLAMMATORY GENES IN SOMATIC ORGANS AS A CONSEQUENCE OF BRAIN DEATH. TRANSPLANT PROC, 1999. 31(1-2): P. 1003-5.
52. VAN DER HOEVEN, J.A., ET AL., RELATIONSHIP BETWEEN DURATION OF BRAIN DEATH AND HEMODYNAMIC (IN) STABILITY ON PROGRESSIVE DYSFUNCTION AND INCREASED IMMUNOLOGIC ACTIVATION OF DONOR KIDNEYS. KIDNEY INT, 2003. 64(5): P. 1874-82.
53. TAKADA, M., ET AL., EFFECTS OF EXPLOSIVE BRAIN DEATH ON CYTOKINE ACTIVATION OF PERIPHERAL ORGANS IN THE RAT. TRANSPLANTATION, 1998. 65(12): P. 1533-42.
54. WILHELM, M.J., ET AL., DONOR BRAIN DEATH AFFECTS TEMPO AND INTENSITY OF ACUTE REJECTION OF RAT CHRONIC ALLOGRAFTS. TRANSPLANT PROC, 1999. 31(1-2): P. 1008-9.
55. PRATSCHKE, J., ET AL., ACUTE REJECTION OF RAT RENAL ALLOGRAFTS IS ACCELERATED BY DONOR BRAIN DEATH. TRANSPLANT PROC, 1999. 31(1-2): P. 874-5.
56. NOVITZKY, D., A.G. ROSE, AND D.K. COOPER, INJURY OF MYOCARDIAL CONDUCTION TISSUE AND CORONARY ARTERY SMOOTH MUSCLE FOLLOWING BRAIN DEATH IN THE BABOON. TRANSPLANTATION, 1988. 45(5): P. 964-6.
57. LABARRERE, C.A., ET AL., VASCULAR TISSUE PLASMINOGEN ACTIVATOR AND THE DEVELOPMENT OF CORONARY ARTERY DISEASE IN HEART-TRANSPLANT RECIPIENTS. N ENGL J MED, 1995. 333(17): P. 1111-6.
58. CHIOLERO, R. AND M. BERGER, ENDOCRINE RESPONSE TO BRAIN INJURY. NEW HORIZ, 1994. 2(4): P. 432-42.
59. HOWIE, A.J., ET AL., MEASUREMENT OF CHRONIC DAMAGE IN THE DONOR KIDNEY AND GRAFT SURVIVAL. TRANSPLANTATION, 2004. 77(7): P. 1058-65.
60. MORRIS, P.J., ET AL., ANALYSIS OF FACTORS THAT AFFECT OUTCOME OF PRIMARY CADAVERIC RENAL TRANSPLANTATION IN THE UK. HLA TASK FORCE OF THE KIDNEY ADVISORY GROUP OF THE UNITED KINGDOM TRANSPLANT SUPPORT SERVICE AUTHORITY (UKTSSA). LANCET, 1999. 354(9185): P. 1147-52.
61. ALEXANDER, J.W., L.E. BENNETT, AND T.J. BREEN, EFFECT OF DONOR AGE ON OUTCOME OF KIDNEY TRANSPLANTATION. A TWO-YEAR ANALYSIS OF TRANSPLANTS REPORTED TO THE UNITED NETWORK FOR ORGAN SHARING REGISTRY. TRANSPLANTATION, 1994. 57(6): P. 871-6.
62. GJERTSON, D.W., A MULTI-FACTOR ANALYSIS OF KIDNEY GRAFT OUTCOMES AT ONE AND FIVE YEARS POSTTRANSPLANTATION: 1996 UNOS UPDATE. CLIN TRANSPL, 1996: P. 343-60.
63. HALLORAN, P.F., A. MELK, AND C. BARTH, RETHINKING CHRONIC ALLOGRAFT NEPHROPATHY: THE CONCEPT OF ACCELERATED SENESCENCE. J AM SOC NEPHROL, 1999. 10(1): P. 167-81.
64. FELLSTROM, B., NONIMMUNE RISK FACTORS FOR CHRONIC RENAL ALLOGRAFT DISFUNCTION. TRANSPLANTATION, 2001. 71(11 SUPPL): P. SS10-6.
65. TERASAKI, P.I., J.M. CECKA, AND D.W. GJERTSON, IMPACT ANALYSIS: A METHOD FOR EVALUATING THE IMPACT OF FACTORS IN CLINICAL RENAL TRANSPLANTATION. CLIN TRANSPL, 1998: P. 437-41.
66. DE FIJTER, J.W., ET AL., INCREASED IMMUNOGENICITY AND CAUSE OF GRAFT LOSS OF OLD DONOR KIDNEYS. J AM SOC NEPHROL, 2001. 12(7): P. 1538-46.
67. FRANCESCHI, C. AND M. BONAFE, CENTENARIANS AS A MODEL FOR HEALTHY AGING. BIOCHEM SOC TRANS, 2003. 31(2): P. 457-61.
68. HARMAN, D., AGING: A THEORY BASED ON FREE RADICAL AND RADIATION CHEMISTRY. J GERONTOL, 1956. 11(3): P. 298-300.
69. CADET, J., ET AL., OXIDATIVE DAMAGE TO DNA: FORMATION, MEASUREMENT, AND BIOLOGICAL SIGNIFICANCE. REV PHYSIOL BIOCHEM PHARMACOL, 1997. 131: P. 1-87.
70. EVANS, M.D. AND M.S. COOKE, FACTORS CONTRIBUTING TO THE OUTCOME OF OXIDATIVE DAMAGE TO NUCLEIC ACIDS. BIOESSAYS, 2004. 26(5): P. 533-42.
71. GRACE, P.A., ISCHAEMIA-REPERFUSION INJURY. BR J SURG, 1994. 81(5): P. 637-47.
72. LI, C. AND R.M. JACKSON, REACTIVE SPECIES MECHANISMS OF CELLULAR HYPOXIA-REOXYGENATION INJURY. AM J PHYSIOL CELL PHYSIOL, 2002. 282(2): P. C227-41.
73. HOEIJMAKERS, J.H., GENOME MAINTENANCE MECHANISMS FOR PREVENTING CANCER. NATURE, 2001. 411(6835): P. 366-74.
74. BOOTSMA, D., KRAEMER, K. H., CLEAVER, J. & HOEIJMAKERS, J. H. J., IN THE METABOLIC AND MOLECULAR BASIS OF INHERITED DISEASE VOL. 1 (EDS SCRIVER, C. R., BEAUDET, A. L., SLY, W. S. & VALLE, D.) 677-703 (MCGRAW-HILL, NEW YORK, 2001). 2001.
75. LEHMANN, A.R., THE XERODERMA PIGMENTOSUM GROUP D (XPD) GENE: ONE GENE, TWO FUNCTIONS, THREE DISEASES. GENES DEV, 2001. 15(1): P. 15-23.

76. DE BOER, J. AND J.H. HOEIJMAKERS, CANCER FROM THE OUTSIDE, AGING FROM THE INSIDE: MOUSE MODELS TO STUDY THE CONSEQUENCES OF DEFECTIVE NUCLEOTIDE EXCISION REPAIR. BIOCHIMIE, 1999. 81(1-2): P. 127-37.
77. DE LAAT, W.L., N.G. JASPERS, AND J.H. HOEIJMAKERS, MOLECULAR MECHANISM OF NUCLEOTIDE EXCISION REPAIR. GENES DEV, 1999. 13(7): P. 768-85.
78. VAN DER HORST, G.T., ET AL., DEFECTIVE TRANSCRIPTION-COUPLED REPAIR IN COCKAYNE SYNDROME B MICE IS ASSOCIATED WITH SKIN CANCER PREDISPOSITION. CELL, 1997. 89(3): P. 425-35.
79. TEBBS, R.S., ET AL., REQUIREMENT FOR THE XRCC1 DNA BASE EXCISION REPAIR GENE DURING EARLY MOUSE DEVELOPMENT. DEV BIOL, 1999. 208(2): P. 513-29.
80. LAVIN, M.F. AND Y. SHILOH, ATAXIA-TELANGIECTASIA: A MULTIFACETED GENETIC DISORDER ASSOCIATED WITH DEFECTIVE SIGNAL TRANSDUCTION. CURR OPIN IMMUNOL, 1996. 8(4): P. 459-64.
81. DU, X., ET AL., TELOMERE SHORTENING EXPOSES FUNCTIONS FOR THE MOUSE WERNER AND BLOOM SYNDROME GENES. MOL CELL BIOL, 2004. 24(19): P. 8437-46.
82. HASTY, P., ET AL., AGING AND GENOME MAINTENANCE: LESSONS FROM THE MOUSE? SCIENCE, 2003. 299(5611): P. 1355-9.
83. DE BOER, J., ET AL., PREMATURE AGING IN MICE DEFICIENT IN DNA REPAIR AND TRANSCRIPTION. SCIENCE, 2002. 296(5571): P. 1276-9.
84. HARLEY, C.B., A.B. FUTCHER, AND C.W. GREIDER, TELOMERES SHORTEN DURING AGEING OF HUMAN FIBROBLASTS. NATURE, 1990. 345(6274): P. 458-60.
85. WONG, J.M. AND K. COLLINS, TELOMERE MAINTENANCE AND DISEASE. LANCET, 2003. 362(9388): P. 983-8.
86. BLASCO, M.A., MAMMALIAN TELOMERES AND TELOMERASE: WHY THEY MATTER FOR CANCER AND AGING. EUR J CELL BIOL, 2003. 82(9): P. 441-6.
87. KRTOLICA, A., STEM CELL: BALANCING AGING AND CANCER. INT J BIOCHEM CELL BIOL, 2005. 37(5): P. 935-41.
88. COLLINS, K. AND J.R. MITCHELL, TELOMERASE IN THE HUMAN ORGANISM. ONCOGENE, 2002. 21(4): P. 564-79.
89. SMOGORZEWSKA, A., ET AL., CONTROL OF HUMAN TELOMERE LENGTH BY TRF1 AND TRF2. MOL CELL BIOL, 2000. 20(5): P. 1659-68.
90. SERRANO, A.L. AND V. ANDRES, TELOMERES AND CARDIOVASCULAR DISEASE: DOES SIZE MATTER? CIRC RES, 2004. 94(5): P. 575-84.
91. SATYANARAYANA, A., ET AL., TELOMERE SHORTENING IMPAIRS ORGAN REGENERATION BY INHIBITING CELL CYCLE RE-ENTRY OF A SUBPOPULATION OF CELLS. EMBO J, 2003. 22(15): P. 4003-13.
92. HARLEY, C.B., TELOMERASE IS NOT AN ONCOGENE. ONCOGENE, 2002. 21(4): P. 494-502.
93. CAMPISI, J., CANCER, AGING AND CELLULAR SENESCENCE. IN VIVO, 2000. 14(1): P. 183-8.
94. MARRONE, A. AND P.J. MASON, DYSKERATOSIS CONGENITA. CELL MOL LIFE SCI, 2003. 60(3): P. 507-17.
95. METCALFE, J.A., ET AL., ACCELERATED TELOMERE SHORTENING IN ATAXIA TELANGIECTASIA. NAT GENET, 1996. 13(3): P. 350-3.
96. HAYFLICK, L. AND P.S. MOORHEAD, THE SERIAL CULTIVATION OF HUMAN DIPLOID CELL STRAINS. EXP CELL RES, 1961. 25: P. 585-621.
97. TOUSSAINT, O., E.E. MEDRANO, AND T. VON ZGLINICKI, CELLULAR AND MOLECULAR MECHANISMS OF STRESS-INDUCED PREMATURE SENESCENCE (SIPS) OF HUMAN DIPLOID FIBROBLASTS AND MELANOCYTES. EXP GERONTOL, 2000. 35(8): P. 927-45.
98. FARAGHER, R.G. AND D. KIPLING, HOW MIGHT REPLICATIVE SENESCENCE CONTRIBUTE TO HUMAN AGEING? BIOESSAYS, 1998. 20(12): P. 985-91.
99. CAMPISI, J., ET AL., CELLULAR SENESCENCE, CANCER AND AGING: THE TELOMERE CONNECTION. EXP GERONTOL, 2001. 36(10): P. 1619-37.
100. HAYFLICK, L., THE CELLULAR BASIS FOR BIOLOGICAL AGING. IN: FINCH, C., HAYFLICK, L. (EDS). HANDBOOK OF THE BIOLOGY OF AGEING. VAN NOSTRAND REINHOLD, NEW YORK, PP. 159-186, 1977.
101. HOUBEN, A., ET AL., ALTERATION OF ENZYMES IN AGEING HUMAN FIBROBLASTS IN CULTURE. I. CONDITIONS FOR THE APPEARANCE OF AN ALTERATION IN GLUCOSE 6-PHOSPHATE DEHYDROGENASE. MECH AGEING DEV, 1984. 25(1-2): P. 23-34.
102. HOUBEN, A., ET AL., ALTERATION OF ENZYMES IN AGEING HUMAN FIBROBLASTS IN CULTURE. II. CONDITIONS FOR THE REVERSIBILITY AND THE MECHANISM OF THE ALTERATION OF GLUCOSE 6-PHOSPHATE DEHYDROGENASE. MECH AGEING DEV, 1984. 25(1-2): P. 35-45.
103. DIMRI, G.P., ET AL., A BIOMARKER THAT IDENTIFIES SENESCENT HUMAN CELLS IN CULTURE AND IN AGING SKIN IN VIVO. PROC NATL ACAD SCI U S A, 1995. 92(20): P. 9363-7.

104. DIERICK, J.F., ET AL., STRESS-INDUCED PREMATURE SENESCENCE AND REPLICATIVE SENESCENCE ARE DIFFERENT PHENOTYPES, PROTEOMIC EVIDENCE. BIOCHEM PHARMACOL, 2002. 64(5-6): P. 1011-7.
105. CAMPISI, J., REPLICATIVE SENESCENCE AND IMMORTALIZATION. IN: STEIN, G.S., BASERGA, R., GIORDANO, A., DENHARDT, D.T. (EDS.). THE MOLECULAR BASIS OF CELL CYCLE AND GROWTH CONTROL. WILEY, NEW YORK, PP.348-373., 1999.
106. SHELTON, D.N., ET AL., MICROARRAY ANALYSIS OF REPLICATIVE SENESCENCE. CURR BIOL, 1999. 9(17): P. 939-45.
107. BODNAR, A.G., ET AL., EXTENSION OF LIFE-SPAN BY INTRODUCTION OF TELOMERASE INTO NORMAL HUMAN CELLS. SCIENCE, 1998. 279(5349): P. 349-52.
108. HARLEY, C.B., TELOMERE LOSS: MITOTIC CLOCK OR GENETIC TIME BOMB? MUTAT RES, 1991. 256(2-6): P. 271-82.
109. CHEN, Q., ET AL., OXIDATIVE DNA DAMAGE AND SENESCENCE OF HUMAN DIPLOID FIBROBLAST CELLS. PROC NATL ACAD SCI U S A, 1995. 92(10): P. 4337-41.
110. VON ZGLINICKI, T., REPLICATIVE SENESCENCE AND THE ART OF COUNTING. EXP GERONTOL, 2003. 38(11-12): P. 1259-64.
111. BOUKAMP, P., BIOLOGICAL CLOCKS IN THE AGEING CELL. IN: T. VON ZGLINICKI, EDITOR, AGING AT THE MOLECULAR LEVEL, KLUWER ACADEMIC PRESS, DORDRECHT, 2003.
112. VON ZGLINICKI, T., TELOMERES AND REPLICATIVE SENESCENCE: IS IT ONLY LENGTH THAT COUNTS? CANCER LETT, 2001. 168(2): P. 111-6.
113. BLACKBURN, E.H., TELOMERE STATES AND CELL FATES. NATURE, 2000. 408(6808): P. 53-6.
114. FERBEYRE, G., ET AL., ONCOGENIC RAS AND P53 COOPERATE TO INDUCE CELLULAR SENESCENCE. MOL CELL BIOL, 2002. 22(10): P. 3497-508.
115. DIMRI, G.P., ET AL., THE BMI-1 ONCOGENE INDUCES TELOMERASE ACTIVITY AND IMMORTALIZES HUMAN MAMMARY EPITHELIAL CELLS. CANCER RES, 2002. 62(16): P. 4736-45.
116. STAMPFER, M.R. AND P. YASWEN, HUMAN EPITHELIAL CELL IMMORTALIZATION AS A STEP IN CARCINOGENESIS. CANCER LETT, 2003. 194(2): P. 199-208.
117. OGRYZKO, V.V., ET AL., HUMAN FIBROBLAST COMMITMENT TO A SENESCENCE-LIKE STATE IN RESPONSE TO HISTONE DEACETYLASE INHIBITORS IS CELL CYCLE DEPENDENT. MOL CELL BIOL, 1996. 16(9): P. 5210-8.
118. ROBLES, S.J. AND G.R. ADAMI, AGENTS THAT CAUSE DNA DOUBLE STRAND BREAKS LEAD TO P16INK4A ENRICHMENT AND THE PREMATURE SENESCENCE OF NORMAL FIBROBLASTS. ONCOGENE, 1998. 16(9): P. 1113-23.
119. D'ADDA DI FAGAGNA, F., ET AL., A DNA DAMAGE CHECKPOINT RESPONSE IN TELOMERE-INITIATED SENESCENCE. NATURE, 2003. 426(6963): P. 194-8.
120. HERSKIND, C. AND H.P. RODEMANN, SPONTANEOUS AND RADIATION-INDUCED DIFFERENTIATIONOF FIBROBLASTS. EXP GERONTOL, 2000. 35(6-7): P. 747-55.
121. SEDELNIKOVA, O.A., ET AL., SENESCING HUMAN CELLS AND AGEING MICE ACCUMULATE DNA LESIONS WITH UNREPAIRABLE DOUBLE-STRAND BREAKS. NAT CELL BIOL, 2004. 6(2): P. 168-70.
122. DUMONT, P., ET AL., OVEREXPRESSION OF APOLIPOPROTEIN J IN HUMAN FIBROBLASTS PROTECTS AGAINST CYTOTOXICITY AND PREMATURE SENESCENCE INDUCED BY ETHANOL AND TERT-BUTYLHYDROPEROXIDE. CELL STRESS CHAPERONES, 2002. 7(1): P. 23-35.
123. FRIPPIAT, C., ET AL., SUBCYTOTOXIC H2O2 STRESS TRIGGERS A RELEASE OF TRANSFORMING GROWTH FACTOR-BETA 1, WHICH INDUCES BIOMARKERS OF CELLULAR SENESCENCE OF HUMAN DIPLOID FIBROBLASTS. J BIOL CHEM, 2001. 276(4): P. 2531-7.
124. SERRA, V. AND T. VON ZGLINICKI, HUMAN FIBROBLASTS IN VITRO SENESCE WITH A DONOR-SPECIFIC TELOMERE LENGTH. FEBS LETT, 2002. 516(1-3): P. 71-4.
125. VON ZGLINICKI, T., OXIDATIVE STRESS SHORTENS TELOMERES. TRENDS BIOCHEM SCI, 2002. 27(7): P. 339-44.
126. VON ZGLINICKI, T., R. PILGER, AND N. SITTE, ACCUMULATION OF SINGLE-STRAND BREAKS IS THE MAJOR CAUSE OF TELOMERE SHORTENING IN HUMAN FIBROBLASTS. FREE RADIC BIOL MED, 2000. 28(1): P. 64-74.
127. SERRA, V., ET AL., EXTRACELLULAR SUPEROXIDE DISMUTASE IS A MAJOR ANTIOXIDANT IN HUMAN FIBROBLASTS AND SLOWS TELOMERE SHORTENING. J BIOL CHEM, 2003. 278(9): P. 6824-30.
128. CAMPISI, J., CELLULAR SENESCENCE AND APOPTOSIS: HOW CELLULAR RESPONSES MIGHT INFLUENCE AGING PHENOTYPES. EXP GERONTOL, 2003. 38(1-2): P. 5-11.
129. XIE, S., ET AL., REACTIVE OXYGEN SPECIES-INDUCED PHOSPHORYLATION OF P53 ON SERINE 20 IS MEDIATED IN PART BY POLO-LIKE KINASE-3. J BIOL CHEM, 2001. 276(39): P. 36194-9.
130. SARETZKI, G., ET AL., SIMILAR GENE EXPRESSION PATTERN IN SENESCENT AND HYPEROXIC-TREATED FIBROBLASTS. J GERONTOL A BIOL SCI MED SCI, 1998. 53(6): P. B438-42.

131. BAYLIS, C. AND B. CORMAN, THE AGING KIDNEY: INSIGHTS FROM EXPERIMENTAL STUDIES. J AM SOC NEPHROL, 1998. 9(4): P. 699-709.
132. BAYLIS, C. AND R. SCHMIDT, THE AGING GLOMERULUS. SEMIN NEPHROL, 1996. 16(4): P. 265-76.
133. EPSTEIN, M., EFFECTS OF AGING ON THE KIDNEY. FED PROC, 1979. 38(2): P. 168-71.
134. ANDERSON, S. AND B.M. BRENNER, EFFECTS OF AGING ON THE RENAL GLOMERULUS. AM J MED, 1986. 80(3): P. 435-42.
135. GOYAL, V.K., CHANGES WITH AGE IN THE HUMAN KIDNEY. EXP GERONTOL, 1982. 17(5): P. 321-31.
136. GOURTSOYIANNIS, N., ET AL., THE THICKNESS OF THE RENAL PARENCHYMA DECREASES WITH AGE: A CT STUDY OF 360 PATIENTS. AJR AM J ROENTGENOL, 1990. 155(3): P. 541-4.
137. KAYSEN, G.A. AND B.D. MYERS, THE AGING KIDNEY. CLIN GERIATR MED, 1985. 1(1): P. 207-22.
138. MELK, A., SENESCENCE OF RENAL CELLS: MOLECULAR BASIS AND CLINICAL IMPLICATIONS. NEPHROL DIAL TRANSPLANT, 2003. 18(12): P. 2474-8.
139. MELK, A., ET AL., EXPRESSION OF P16INK4A AND OTHER CELL CYCLE REGULATOR AND SENESCENCE ASSOCIATED GENES IN AGING HUMAN KIDNEY. KIDNEY INT, 2004. 65(2): P. 510-20.
140. SERRANO, M., ET AL., ROLE OF THE INK4A LOCUS IN TUMOR SUPPRESSION AND CELL MORTALITY. CELL, 1996. 85(1): P. 27-37.
141. PALMERO, I., ET AL., ACCUMULATION OF P16INK4A IN MOUSE FIBROBLASTS AS A FUNCTION OF REPLICATIVE SENESCENCE AND NOT OF RETINOBLASTOMA GENE STATUS. ONCOGENE, 1997. 15(5): P. 495-503.
142. KAMIJO, T., ET AL., TUMOR SUPPRESSION AT THE MOUSE INK4A LOCUS MEDIATED BY THE ALTERNATIVE READING FRAME PRODUCT P19ARF. CELL, 1997. 91(5): P. 649-59.
143. WRIGHT, W.E. AND J.W. SHAY, TELOMERE DYNAMICS IN CANCER PROGRESSION AND PREVENTION: FUNDAMENTAL DIFFERENCES IN HUMAN AND MOUSE TELOMERE BIOLOGY. NAT MED, 2000. 6(8): P. 849-51.
144. MELK, A., ET AL., TELOMERE SHORTENING IN KIDNEYS WITH AGE. J AM SOC NEPHROL, 2000. 11(3): P. 444-53.
145. MELK, A., ET AL., CELL SENESCENCE IN RAT KIDNEYS IN VIVO INCREASES WITH GROWTH AND AGE DESPITE LACK OF TELOMERE SHORTENING. KIDNEY INT, 2003. 63(6): P. 2134-43.
146. HASTINGS, R., ET AL., RAPID TELOMERE ATTRITION IN CARDIAC TISSUE OF THE AGEING WISTAR RAT. EXP GERONTOL, 2004. 39(5): P. 855-7.
147. LINDSEY, J., ET AL., IN VIVO LOSS OF TELOMERIC REPEATS WITH AGE IN HUMANS. MUTAT RES, 1991. 256(1): P. 45-8.
148. VAZIRI, H., ET AL., EVIDENCE FOR A MITOTIC CLOCK IN HUMAN HEMATOPOIETIC STEM CELLS: LOSS OF TELOMERIC DNA WITH AGE. PROC NATL ACAD SCI U S A, 1994. 91(21): P. 9857-60.
149. ALLSOPP, R.C., ET AL., TELOMERE SHORTENING IS ASSOCIATED WITH CELL DIVISION IN VITRO AND IN VIVO. EXP CELL RES, 1995. 220(1): P. 194-200.
150. CHANG, E. AND C.B. HARLEY, TELOMERE LENGTH AND REPLICATIVE AGING IN HUMAN VASCULAR TISSUES. PROC NATL ACAD SCI U S A, 1995. 92(24): P. 11190-4.
151. CHERIF, H., ET AL., AGEING AND TELOMERES: A STUDY INTO ORGAN- AND GENDER-SPECIFIC TELOMERE SHORTENING. NUCLEIC ACIDS RES, 2003. 31(5): P. 1576-83.
152. DEMERATH, E.W., ET AL., TELOMERES AND TELOMERASE IN THE FETAL ORIGINS OF CARDIOVASCULAR DISEASE: A REVIEW. HUM BIOL, 2004. 76(1): P. 127-46.
153. OH, H., ET AL., TELOMERE ATTRITION AND CHK2 ACTIVATION IN HUMAN HEART FAILURE. PROC NATL ACAD SCI U S A, 2003. 100(9): P. 5378-83.
154. MINAMINO, T., ET AL., ENDOTHELIAL CELL SENESCENCE IN HUMAN ATHEROSCLEROSIS: ROLE OF TELOMERE IN ENDOTHELIAL DYSFUNCTION. CIRCULATION, 2002. 105(13): P. 1541-4.
155. PARADIS, V., ET AL., REPLICATIVE SENESCENCE IN NORMAL LIVER, CHRONIC HEPATITIS C, AND HEPATOCELLULAR CARCINOMAS. HUM PATHOL, 2001. 32(3): P. 327-32.
156. WIEMANN, S.U., ET AL., HEPATOCYTE TELOMERE SHORTENING AND SENESCENCE ARE GENERAL MARKERS OF HUMAN LIVER CIRRHOSIS. FASEB J, 2002. 16(9): P. 935-42.
157. KITADA, T., ET AL., TELOMERE SHORTENING IN CHRONIC LIVER DISEASES. BIOCHEM BIOPHYS RES COMMUN, 1995. 211(1): P. 33-9.
158. AKIYAMA, M., ET AL., SHORTENING OF TELOMERES IN RECIPIENTS OF BOTH AUTOLOGOUS AND ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION. BONE MARROW TRANSPLANT, 2000. 25(4): P. 441-7.
159. WYNN, R.F., ET AL., ACCELERATED TELOMERE SHORTENING IN YOUNG RECIPIENTS OF ALLOGENEIC BONE-MARROW TRANSPLANTS. LANCET, 1998. 351(9097): P. 178-81.
160. MELK, A., ET AL., INCREASED EXPRESSION OF SENESCENCE-ASSOCIATED CELL CYCLE INHIBITOR P16INK4A IN DE-

TERIORATING RENAL TRANSPLANTS AND DISEASED NATIVE KIDNEY. AM J TRANSPLANT, 2005. 5(6): P. 1375-82.
161. FERLICOT, S., ET AL., THE ROLE OF REPLICATIVE SENESCENCE IN CHRONIC ALLOGRAFT NEPHROPATHY. HUM PATHOL, 2003. 34(9): P. 924-8.
162. CHKHOTUA, A.B., ET AL., INCREASED EXPRESSION OF P16(INK4A) AND P27(KIP1) CYCLIN-DEPENDENT KINASE INHIBITOR GENES IN AGING HUMAN KIDNEY AND CHRONIC ALLOGRAFT NEPHROPATHY. AM J KIDNEY DIS, 2003. 41(6): P. 1303-13.
163. CHKHOTUA, A.B., ET AL., INCREASED EXPRESSION OF P21 (WAF1/CIP1) CYCLIN-DEPENDENT KINASE (CDK) INHIBITOR GENE IN CHRONIC ALLOGRAFT NEPHROPATHY CORRELATES WITH THE NUMBER OF ACUTE REJECTION EPISODES. TRANSPL INT, 2003. 16(8): P. 502-6.
164. MUNRO, J., ET AL., HUMAN FIBROBLAST REPLICATIVE SENESCENCE CAN OCCUR IN THE ABSENCE OF EXTENSIVE CELL DIVISION AND SHORT TELOMERES. ONCOGENE, 2001. 20(27): P. 3541-52.
165. TANNER, F.C., ET AL., DIFFERENTIAL EFFECTS OF THE CYCLIN-DEPENDENT KINASE INHIBITORS P27(KIP1), P21(CIP1), AND P16(INK4) ON VASCULAR SMOOTH MUSCLE CELL PROLIFERATION. CIRCULATION, 2000. 101(17): P. 2022-5.
166. JOOSTEN, S.A., ET AL., TELOMERE SHORTENING AND CELLULAR SENESCENCE IN A MODEL OF CHRONIC RENAL ALLOGRAFT REJECTION. AM J PATHOL, 2003. 162(4): P. 1305-12.

Chapter 3

Congenital DNA repair deficiency results in protection against renal ischemia reperfusion injury in mice

D Susa, JR Mitchell[#], M Verweij, M van de Ven[#], HP Roest, S van den Engel, IM Bajema[‡], K Mangundap, JNM IJzermans, JHJ Hoeijmakers[#], RWF de Bruin

Department of Surgery, Department of Genetics[#], Erasmus Medical Center, Rotterdam, The Netherlands
Department of Pathology[‡], Leiden University Medical Center, Leiden, The Netherlands

These authors (D Susa, JR Mitchell) contributed equally to this work

Aging Cell 2009; 8 (2): 192-200

ABSTRACT

Cockayne syndrome and other segmental progerias with inborn defects in DNA repair mechanisms are thought to be due in part to hypersensitivity to endogenous oxidative DNA damage. The accelerated aging-like symptoms of this disorder include dysmyelination within the central nervous system, progressive sensineuronal hearing loss and retinal degeneration. We tested the effects of congenital nucleotide excision DNA repair deficiency on acute oxidative stress sensitivity in vivo. Surprisingly, we found mouse models of Cockayne syndrome less susceptible than wild type animals to surgically induced renal ischemia reperfusion injury, a multifactorial injury mediated in part by oxidative damage. Renal failure-related mortality was significantly reduced in *Csb* $^{-/-}$ mice, kidney function was improved and proliferation was significantly higher in the regenerative phase following ischemic injury. Protection from ischemic damage correlated with improved baseline glucose tolerance and insulin sensitivity and a reduced inflammatory response following injury. Protection was further associated with genetic ablation of a different Cockayne syndrome-associated gene, *Csa*. Our data provide the first functional in vivo evidence that congenital DNA repair deficiency can induce protection from acute stress in at least one organ. This suggests that while specific types of unrepaired endogenous DNA damage may lead to detrimental effects in certain tissues, they may at the same time elicit beneficial adaptive changes in others and thus contribute to the tissue specificity of disease symptoms.

INTRODUCTION

The case for the involvement of oxidative damage to macro-molecules in the etiology of aging, aging-related disorders and so-called 'premature aging' disorders (here referred to as segmental progerias) is compelling. The free radical theory as originally proposed by Harman [1] and extended in recent years [2] posits that reactive oxygen species (ROS), by-products of endogenous cellular metabolism, inflict damage on lipids, protein and DNA. Despite defence systems to prevent or repair such damage, oxidized macromolecules accumulate over time in vivo and are thought to underlie both normal and pathological aging. When combined with congenital defects in certain of these defence systems, for example, genome stability mechanisms, endogenous oxidative damage may also contribute to the symptoms of segmental progerias, which display some but not all of the characteristics of normal aging in accelerated or exacerbated forms [3].

Nucleotide excision DNA repair (NER) is one such defence system that removes a range of damages, including helix distorting lesions induced either by exogenous ultraviolet radiation (e.g. pyrimidine dimers) or endogenous oxidative radicals (e.g. cyclopurines) [4]. Two basic modes of lesion recognition have been defined. In the first, lesions occurring anywhere in the genome are recognized by damage-binding proteins such as the XPC-HR23A/B-CEN1 complex. This triggers the assembly of the multiprotein NER machinery, which functions via a cut and patch mechanism to remove the damage and fill the remaining single-strand gap [5]. In the second mode of recognition, transcription-coupled (TC)-NER, lesions that block an elongating RNA polymerase trigger assembly of the NER machinery in a process that depends on the chromatin remodelling protein CSB [6] and a ubiquitin ligase complex containing the TC-NER specific protein CSA [7, 8].

Defects in CSA or CSB can give rise to Cockayne syndrome, a severe neurodevelopmental disease marked by photosensitivity (but curiously without skin cancer predisposition), dysmyelination within the central nervous system, progressive sensineuronal hearing loss, retinal degeneration and cachectic dwarfism resulting in an aged appearance [9]. Mouse models lacking Csa or Csb function recapitulate some progeroid characteristics of the human disease, including cachexia and progressive loss of photoreceptor cells, but with a normal lifespan and an overall milder phenotype than in humans [10-12]. In support of the role of oxidative stress hypersensitivity in Cockayne syndrome etiology, an increase in photoreceptor cell loss following whole-body ionizing radiation was observed in both *Csa*$^{-/-}$ and *Csb*$^{-/-}$ mice [13]. Thus, at least particular CS cell types appear hypersensitive to the effects of oxidative DNA damage, a property that may underlie tissue-specific disease phenotypes.

Contrary to the notion of hypersensitivity to oxidative stress in Cockayne syndrome is the observation that mouse models of Cockayne syndrome and related NER-deficient segmental progerias (NER progerias) display characteristics of hypopituitary dwarf mutants or dietary restricted wild type mice [14-18]. In dwarf and dietary restricted mice, these phenotypes, including reduced body weight and temperature, hypoglycemia, hypoinsulinemia and reduced serum IGF-1, are correlated not only with extended longevity but also resistance to acute oxidative stress [19]. In progeroid NER mice, these phenotypes have been interpreted as an adaptive response to unrepaired endogenous DNA damage engaged to protect from further oxidative stress injury [20]. One important prediction of this interpretation is that mice should be resistant rather than hyper-

sensitive to acute oxidative stress.

Ischemia reperfusion injury is a complex insult initiated by loss of blood flow to an organ. During the ischemic period, the lack of molecular oxygen as an electron acceptor in oxidative phosphorylation prevents ATP generation and compromises processes with high energy demand, such as maintenance of ion gradients across intracellular membranes. Reinitiation of oxygenated blood flow, or reperfusion, results in inappropriate activation of cellular oxidases and ROS generation, which can affect not only the reperfused organ but distant sites in the body as well [21]. Following reperfusion, a maladaptive inflammatory response mediated in part by the release of ROS from neutrophils infiltrating the tissue causes further injury [22]. In the kidney, ischemia reperfusion injury is associated with cell death primarily in the stripe between the cortex and medulla consisting mainly of tubular epithelial cells via necrosis or apoptosis, depending on the severity of the insult [23]. Recovery and a return to proper kidney function depends on regeneration of the tubular epithelial cells and remodelling of the renal tubules, a process which takes place in the days and weeks following the injury.

We used surgically induced renal ischemia reperfusion injury to test whether mouse models of Cockayne syndrome are susceptible to acute oxidative stress, as predicted by photoreceptor sensitivity to ionizing radiation, or protected from it, as predicted by physiological similarities between other short-lived progeroid NER mice and long-lived, stress resistant mice. Here we report resistance to renal ischemia reperfusion injury in mouse models of Cockayne syndrome.

RESULTS

Warm ischemia was induced in the left kidney of male wild type and *Csb*$^{-/-}$ (subsequently referred to as WT and CSB, respectively) mice for 37 min by clamping the renal artery/vein with a non-traumatic surgical clamp. The 37-min time point was chosen based on the results of preliminary experiments in which ischemia times were titrated between 30 and 45 min. Following clamp release, the undamaged right kidney was removed so that animal survival depended on the function of the damaged kidney; mice entirely lacking kidney function build up toxic compounds in their blood and die within 2–4 days. Survival of CSB mice was significantly higher than WT (Figure 1A). On post-operative day (POD) 7, survival was 56% in CSB mice but only 30% in the WT group (log rank score $P < 0.0033$; n = 24 CSB, 40 WT).

Kidney function was determined by measuring the concentrations of urea and creatinine in the serum. High levels of these waste products indicate an inability of the kidney to remove them from the blood and thus correlate inversely with kidney function. Interestingly, pre-operative urea and creatinine values were slightly but significantly lower in CSB mice than in WT mice (urea 8.5 ± 1.3 mmol L^{-1} (CSB) vs. 9.7 ± 1.2 mmol L^{-1} (WT), P = 0.0007; creatinine 95 ± 17 µmol L^{-1} (CSB) vs. 122 ± 34 µmol L^{-1} (WT), P = 0.002). Following ischemia reperfusion injury, these markers of kidney function rose with similar kinetics, suggesting maximal dysfunction on POD 2 and a return to function beginning on POD 3. However, average values were significantly lower in CSB animals on POD 1 for urea and on PODs 1 and 2 for creatinine (Figure 1B,C), consistent with better survival in the CSB group.

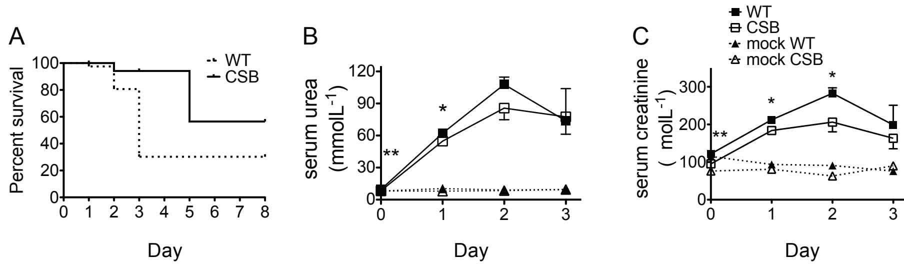

Figure 1. CSB mice are better protected against warm renal ischemia reperfusion injury than WT. (A) Kaplan-Meier survival curves of CSB (n = 24) and WT (n = 40) animals; a log rank score of P < 0.0033 indicates a highly urea (B) and creatinine (C). Statistically significant differences in magnitude observed at individual time treatment that did not involve ischemia are indicated.

We next analysed cell death at various time points after the injury to look for differences that could explain the observed survival and functional benefits in CSB animals vs. WT controls. We used histology to score for acute tubular necrosis, the major form of cell death due to this type of injury (Figure 2A). In both groups, acute tubular necrosis peaked on POD2; in the CSB group, average total cell death was lower at all time points, reaching statistical significance on PODs 1 and 2. The high mortality due to kidney dysfunction in the wild type group prevented meaningful comparisons beyond POD 3.

Following cell death and clearance of cellular debris by shedding into the lumen of the tubules, kidneys damaged by ischemia reperfusion injury may undergo a regenerative phase in which cellular proliferation can be observed. Since the survival benefit of the CSB mice was likely only partially explained by the lower cell death on PODs 1 and 2, we next asked whether regeneration of the kidney was enhanced. We used three different assays to gauge this parameter: histology, or the presence of mitotic figures in H&E stained sections; immunohistochemistry against the proliferating cell nuclear antigen (PCNA); and PCNA immunoblotting of total kidney homogenates to assess total levels of this protein. In both groups, mitotic figures were first seen on POD 3, with significantly higher scores in CSB mice (Figure 2B; P = 0.025). We next looked at PCNA on the single-cell level by immunohistochemistry. The number of PCNA positive cells was significantly increased in the CSB group on POD 1 (Figure 2C; P = 0.035), possibly indicative of its additional role in DNA repair. We further analysed PCNA levels in total kidney homogenates by immunoblot. Although pre-operative PCNA levels were similar between groups, significantly higher levels of PCNA were observed on POD 3 in the CSB group (Figure 2D; P = 0.042). Taken together, these results are consistent with enhanced proliferation leading to better regeneration and survival in CSB vs. WT animals.

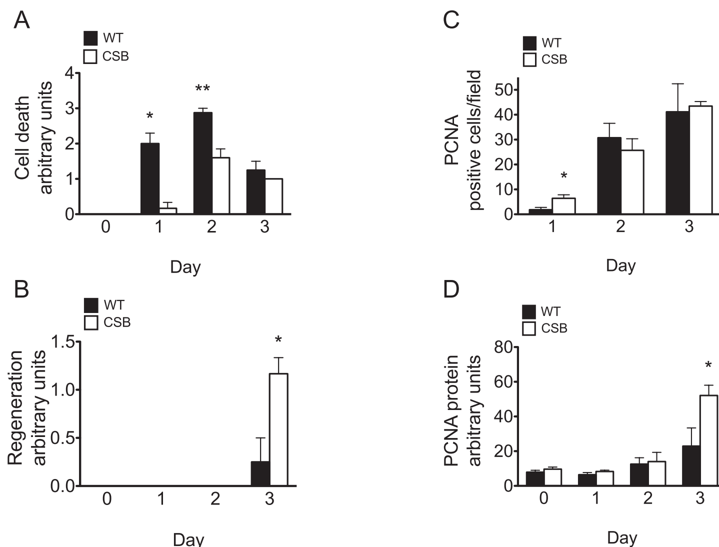

Figure 2. Cell loss and regeneration following 37 min warm renal ischemia reperfusion injury. (A) Cell death as determined by H&E-stained paraffin sections on the indicated day following IR. Day 0 kidneys were contralateral kidneys harvested immediately after clamp release. (B–D) Regeneration as determined by mitotic index in H&E stained sections (B); relative number of cells expressing PCNA as determined by IHC (C); and total PCNA protein level as determined by immunoblotting on the indicated days following ischemia reperfusion injury (D). Statistically significant differences between CSB and WT on a given day are indicated by asterisks (**P < 0.01, *P < 0.05).

Previously, it was reported that wild type C57BL/6 mice subjected to 30–50 min of warm ischemia to one kidney followed by contralateral nephrectomy had a 1-week survival of approximately 80%, with no statistically significant differences between ischemia times [24]. We consistently found mortality of approximately 70% due to kidney failure following 30–37 min warm ischemia (Figure 1A and data not shown), making it difficult to study the course of organ recovery beyond POD3. To circumvent this problem, we repeated our experiments in WT and CSB mice using 25 min of warm renal ischemia. With this amount of ischemic damage, survival was 100% in both WT and CSB groups (data not shown). However, 25 min of ischemia resulted in significantly less kidney dysfunction in mice lacking the CSB protein relative to WT controls at all time points following ischemic damage as measured by serum urea (Figure 3A, top). A similar trend was seen for creatinine (Figure 3A, bottom).

Analysis of total cell death by histology revealed a significant elevation above preoperative values in CSB and WT (although less than after 37 min warm ischemia) on all PODs examined, but no significant differences between genotypes (data not shown). Despite similar low amounts of cell death between groups, we observed large differences in proliferation following 25 min warm renal ischemia. As with 37 min ischemia, proliferation was elevated in CSB vs. WT mice. Regeneration scored by mitotic figures in histological sections was first seen in wild type mice on POD 3, while regeneration was already significantly elevated relative to baseline in CSB mice on PODs

2 and 3 (Figure 3B; P = 0.035 and P = 0.021, respectively). The number of PCNA positive cells on PODs 1 and 2 was also significantly increased in the CSB group (Figure 3C; P = 0.005 and P = 0.002, respectively). Because PCNA is involved in DNA repair as well as proliferation, we stained sections for an additional proliferative marker, Ki-67, which is expressed during all active phases of the cell cycle (G1, S, G2 and mitosis) but is absent from resting cells (G0). Ki-67 positive cells were also increased in CSB mice, with a maximum on POD 2 (Figure 3D; P = 0.016).
The cyclin-dependant kinase inhibitor p21, which binds stoichiometrically to PCNA and inhibits its action in DNA replication, is a key regulator of proliferation following renal ischemic injury. Mice lacking p21 display hyperproliferation and greatly reduced survival [24]. We tested p21 protein levels by immunohistochemistry (Figure 3E). Consistent with increased proliferation in CSB relative to wild type on PODs 1 and 2, the number of p21 positive cells was significantly reduced in CSB mice (POD 2, P = 0.016). Interestingly, p21 staining was equal in wild type and CSB on POD3, coincident with a decline in the first wave of proliferation following injury.

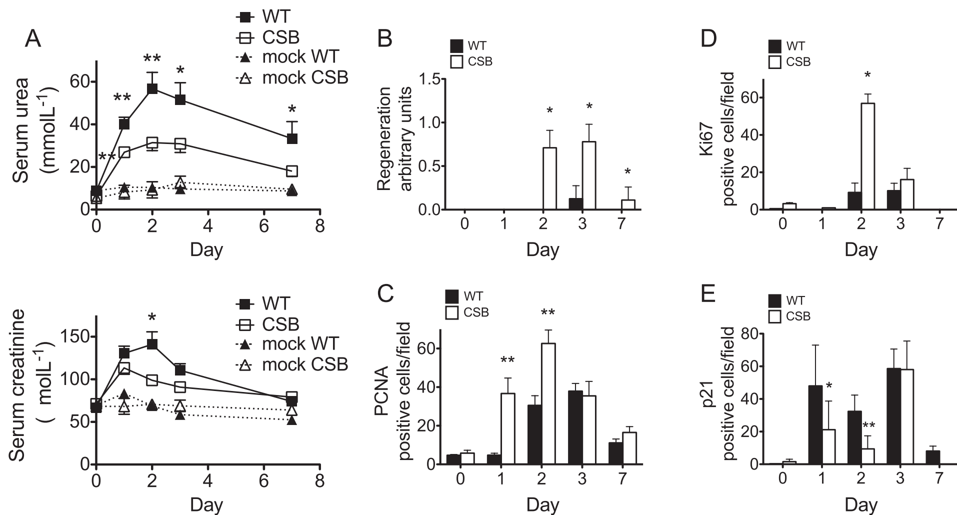

Figure 3. Kidney function, cell death and regeneration following 25 min warm renal ischemia reperfusion injury in CSB and WT animals. (A) Kidney function of the indicated genotypes following IRI or mock treatment as determined by concentration of serum urea (top) or creatinine (bottom). Statistically significant differences between CSB and WT on a given day are indicated by asterisks (**P < 0.01, *P < 0.05). (B–E) Regeneration as indicated by mitotic figures in H&E stained sections (B) and immunohistochemistry against PCNA (C), Ki67 (D) and p21 (E) as indicated. Statistically significant differences between CSB and WT on a given day are indicated by asterisks (**P < 0.01, *P < 0.05).

We next turned to potential mechanisms of protection against renal ischemia reperfusion injury specific to CSB animals. Addition of antioxidants is a proven way to ameliorate the effects of this injury [25]. Furthermore, antioxidant capacity is increased in multiple organs in both genetic dwarfism and dietary restriction, a property thought to contribute to stress resistance and increased longevity in these mice [19]. We thus looked for evidence of increased antioxidant capacity on the level of gene expression. We used quantitative RT-PCR over a time course before and after renal ischemia reperfusion injury to analyse antioxidant defence capabilities as represented by the steady state levels of candidate mRNAs including superoxide dismutase-1, glutathione reductase and hemoxygenase-1 (Figure 4A and data not shown).

At baseline, steady state amounts of these mRNAs were not significantly different between WT and CSB groups. Similarly, over a 24-h period following 25 or 37 min warm renal ischemia, some of these genes (e.g. hemoxygenase-1) were significantly upregulated above baseline but none were consistently, significantly differentially regulated between CSB and WT groups (Figure 4A and data not shown).

Another proven way to reduce the effects of ischemia reperfusion injury is to prevent the inflammatory response that follows tissue injury, for example, by neutralization of cellular adhesion molecules that serve to recruit neutrophils to the site of tissue injury by genetic or antibody-based methods [26]. We examined the expression of P-selectin and ICAM-1 – endothelial adhesion molecules that are upregulated upon tissue injury and whose expression correlates negatively with survival and functional outcomes. We observed a significant increase in both markers following renal IRI above baseline levels; this increase, however, was less in the CSB animals (Figure 4A). An area-under-the-curve analysis revealed significant differences in expression of both P-selectin (P = 0.02) and ICAM-1 (P = 0.05) following renal ischemia reperfusion injury, consistent with less damage subsequent to the reperfusion and a better outcome.

Finally, enhanced sensitivity to the effects of insulin on glucose metabolism is a property shared by dwarf and dietary restricted mice that had been proposed to underlie longevity benefits observed in these models [19]. We chose to analyse glucose metabolism by performing both glucose tolerance and insulin sensitivity tests in overnight fasted CSB and WT mice. We found a significant increase in the ability of CSB animals to clear glucose from the circulation in response to a bolus injection of insulin or glucose, respectively (Figure 4B). It should be noted that despite the slight but significant elevation of fasting glucose levels observed at baseline in CSB animals subject to the glucose tolerance test, no significant difference was observed in the insulin tolerance test between CSB and WT (P = 0.34) nor in the combined data set (P = 0.41). This lack of a significant difference is consistent with previous reports of fed glucose levels of single mutant CS and XPCS mice at postnatal day 15 prior to weaning, while in more severe XPA-deficient double mutant animals hypoglycemia was observed [16, 17].

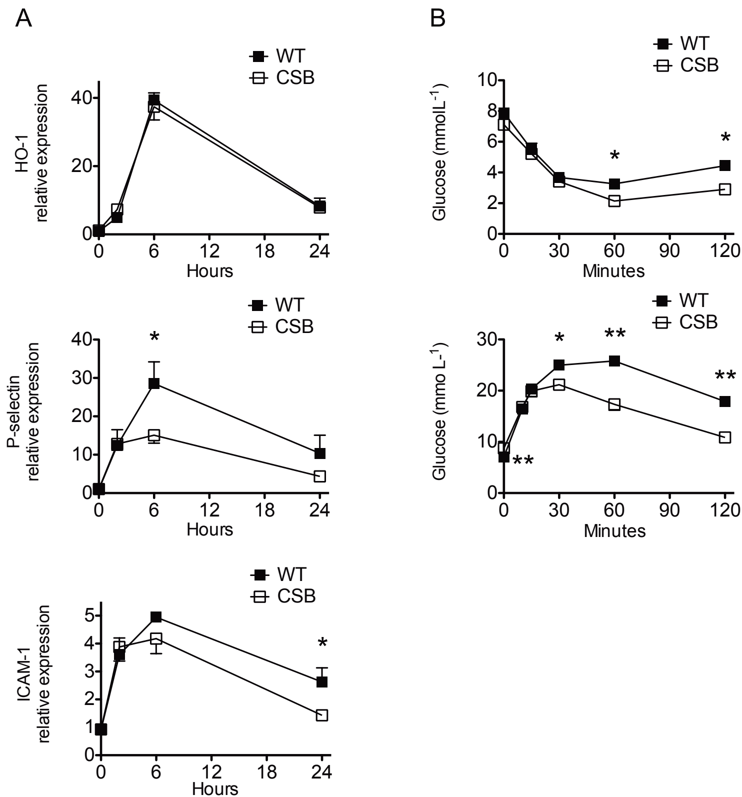

Figure 4. Mechanisms of reduced susceptibility to renal ischemia reperfusion injury in CSB vs. WT mice. (A) Time course of hemeoxygenase-1 (HO-1), P-selectin and ICAM-1 mRNA expression as indicated in the kidney following 37 min renal ischemia reperfusion injury using quantitative real time PCR. Expression levels at all time points were set relative to the WT group at t = 0 hours. Statistically significant differences between genotypes at a given time point day are indicated by an asterisk (*P < 0.05). (B) Improved insulin sensitivity and glucose clearance in CSB vs. WT mice as indicated. Whole blood glucose levels at the indicated time points following intraperitoneal injection of insulin (n = 11 WT, 7 CSB) or glucose (n = 9 WT, 5 CSB) into overnight fasted animals. Statistically significant differences between genotypes at a given time point day are indicated by asterisks (**P < 0.01, *P < 0.05).

Cockayne syndrome can also be caused by defects in the CSA gene. In mice, *Csa*$^{-/-}$ (subsequently referred to as CSA) mice are nearly indistinguishable from CSB mice [11]. We tested the resistance of CSA mice to renal ischemia reperfusion injury and found evidence of protection against both 25 and 37 min warm ischemia in CSA mice vs. WT controls at the level of kidney function (Figure 5A).

No sex bias has been previously reported for Cockayne syndrome [9] or for mouse models of this disease. To see whether the protective effects we observed are specific to males, we tested CSB females for resistance to 37 min of warm renal ischemia. Female mice are in general less susceptible to this injury than males [27], and as expected we observed less evidence of kidney dysfunction-related mortality following 37 min warm ischemia in females than in males. However, CSB females displayed evidence of increased resistance to injury, including significantly lower urea and creatinine in the serum 24 h after reperfusion (Figure 5B).

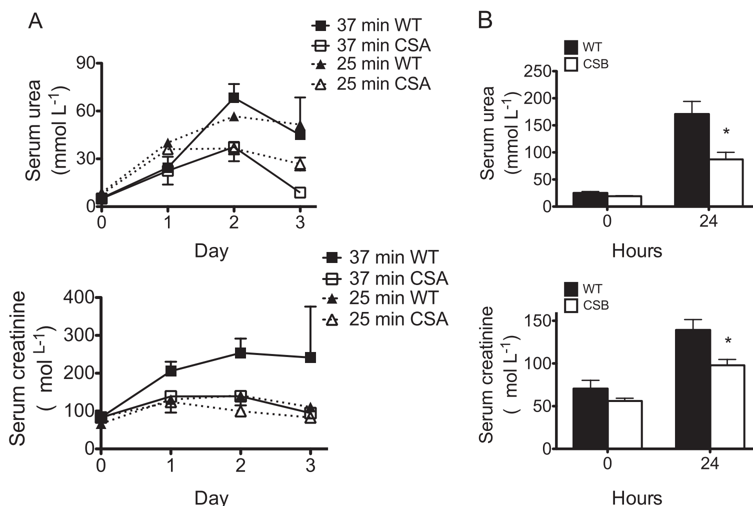

Figure 5. Protection from renal ischemia reperfusion correlates with genetic defects in TC-NER and extends to CSB females. (A) Functional response of TC-NER deficient CSA vs. WT animals to 25 and 37 min warm IRI according to serum urea (top) and creatinine (bottom) values on the indicated days after IRI. Statistically significant differences between genotypes at a given time point day are indicated by asterisks (**$P < 0.01$, *$P < 0.05$). (B) Functional response of WT vs. CSB females to 37 min warm renal IRI as measured by serum urea (top) and creatinine (bottom) values prior to and 24 h after injury.

DISCUSSION

Although the genetic lesions causative of Cockayne syndrome are known, the molecular defect(s) leading to the observed symptoms remains unclear. The prominent inability to repair UV-induced DNA lesions in Cockayne cells from both man and mouse models has led to the hypothesis that hypersensitivity to DNA damage, presumably oxidative in nature, is primarily responsible for disease symptoms. Consistent with this, CSB mice are hypersensitive to the effects of whole-body ionizing irradiation, a potent oxidative stress [28], and both CSA and CSB mice demonstrate an increase in photoreceptor cell loss following ionizing radiation [13]. However, primary CSB cells [16] as well as immortalized CSA cells [29] are not hypersensitive to oxidative stress.

A number of recent reports have described overlap between short-lived segmental progeroid NER models and long-lived hypopituitary dwarfs or dietary restricted wild type mice on the level

of physiology and gene expression [14-18, 20]. These phenotypes have been interpreted as an adaptive response to genotoxic stress overlapping with the starvation response. A previously untested prediction of this interpretation is increased resistance rather than hypersensitivity to acute oxidative stress in these models.

Consistent with this prediction, we showed here that mouse models of Cockayne syndrome deficient in either Csa or Csb are less susceptible to renal ischemia reperfusion injury than wild type mice. Susceptibility was measured in terms of animal survival, kidney function and histological evidence of damage and organ regeneration. In all cases, CSB mice did significantly better than WT mice. On the molecular level, we observed less upregulation of the cell-cycle inhibitor p21 after injury in CSB mice, consistent with less damage and/or better proliferative potential. Upregulation of inflammatory markers ICAM-1 and P-selectin were also significantly reduced following injury in CSB mice, consistent with either reduced damage and/or a dampened ability to mount an inflammatory response. Finally, improved glucose tolerance and insulin sensitivity at baseline were observed in overnight fasted CSB animals relative to WT controls. Taken together with previous studies demonstrating hypersensitivity to ionizing radiation in certain tissues, these data suggest that hypersensitivity and resistance to oxidative stress can coexist in the same animal, depending on the tissue or cell type involved.

Although the mechanism of protection against acute stress in the long-lived dwarf or dietary restricted models is not known, a number of plausible candidate mechanisms have been identified. These include increased antioxidant capacity, reduced inflammation and improved glucose homeostasis due to heightened insulin sensitivity [19]. The induction of genes including heme-oxygenase-1 upon ischemic injury demonstrated that the capacity of CSB animals to mount a transcriptional response is normal despite a defect in the repair of RNA polymerase-stalling bulky DNA lesions. However, we did not observe any differences in antioxidant capacity on the level of gene expression between WT and CSB mice. We did find a difference in the upregulation of two markers of inflammation, P-selectin and ICAM-1. Reduced inflammation is associated with improved outcome following renal ischemia [22], suggesting that these differences may in part underlie the stress resistance observed in the CSB model.

Glucose tolerance and insulin sensitivity tests were both consistent with improved response of CSB animals to the effects of insulin. Improved insulin sensitivity is associated with extended longevity in various models including genetic dwarfism and dietary restriction and is usually associated with reduced IGF-1 and improved glucose metabolism. How improved insulin sensitivity might underlie protection from acute stress observed here, or extended longevity observed in other models, remains unknown. As insulin and insulin-like growth factor signalling are pro-survival, and growth factor delivery can improve outcome after ischemic injury to other organs including the brain [30], we speculate that increased sensitivity to these growth factors may result in both better cell survival as well as improved proliferation following ischemia reperfusion injury.

Which defective biochemical activity (or activities) of CSB is causative of disease phenotypes, possibly including protection from renal ischemic insult? We speculate that a defect in TC-NER is the likely culprit based on the following genetic argument. CSB and other proteins implicated in Cockayne syndrome are multifunctional and have roles in various cellular processes, defects

in any of which could in principle give rise to disease symptoms. Nonetheless the symptoms of Cockayne syndrome are remarkably similar regardless of the underlying genetic lesion, particularly in mice including CSA, CSB, XPCS or TTD models in an XPA deficient background, as well as in XPG, XPF and ERCC1 single mutant animals [20]. We speculate that this common phenotype likely stems from a defect in a shared process in which each of these proteins participates directly. To date, the only common pathway in which each of these multifunctional proteins has a direct role is TC-NER. It should be noted, however, that the presumed unrepaired lesion or repair intermediate remains to be identified.

We propose a model in which a congenital DNA repair deficiency results in activation of systemic protective mechanisms including a reduced inflammatory response and improved insulin sensitivity. These mechanisms would then lend resistance to certain forms of acute stress in some tissues (renal ischemia reperfusion injury to the kidney, as shown here) but not others (ionizing radiation in the photoreceptor layer of the eye [13]). We can envision at least two distinct mechanisms by which this organ/tissue specificity could occur. Based on the neuronal phenotypes in Cockayne patients and mouse models, TC-NER deficiency could in principle result in neuronal deficiencies causing a state of real or perceived dietary restriction, for example, through defective function of neurons involved in nutrient sensing, or neuronal control of the gut leading to malabsorption. Extended periods of dietary restriction in wild type mice have been shown to protect against ischemic damage to organs including the heart [31] and the brain [32]. Alternately, unrepaired TC-NER substrates in the genome may elicit an adaptive stress response similar to dietary restriction but in the absence of reduced food intake. Consistent with this latter interpretation is the adaptive response involving transient alterations in glucose homeostasis and serum IGF-1 levels observed in a related TC-NER deficient mouse model during the potentially stressful period of postnatal development [16].

Hormesis is a common biological phenomenon in which exposure to a low intensity stressor induces a general adaptive response that has net beneficial effects on the cellular and/or organismal level, including protection against subsequent, higher-dose exposures as well as to different types of stress [33, 34]. Dietary restriction has been proposed to act as a mild stressor that extends longevity through hormetic mechanisms [35, 36]. Interestingly, ischemic preconditioning, a procedure used to protect against ischemic insult that entails brief period(s) of ischemia prior to a longer ischemia time, is also thought to function via hormesis [37]. Our data suggest that specific types of unrepaired endogenous DNA lesions may also be hormetic in nature. The interplay between tissue-specific sensitivities to endogenous DNA damage and the resulting adaptive responses may underlie the complex phenotypes observed in segmental disorders such as Cockayne syndrome. Having identified such unexpected benefits associated with DNA repair deficiency, further elucidation of underlying mechanisms may allow exploitation of the benefits without suffering the severe complications associated with congenital DNA repair insufficiencies.

EXPERIMENTAL PROCEDURES

Animals

Animals were allowed free access to food and water throughout the experiments. All experiments were performed with the approval of the appropriate ethical board. Male *Csb*$^{-/-}$ [12] and *Csa*$^{-/-}$ [11] mice in a C57BL/6 background were bred at the animal facility of the Erasmus Medical Centre; C57BL/6 J mice were purchased from Harlan, Horst, the Netherlands.

Ischemia model

Mice between 12 and 16 weeks of age were anaesthetized by isoflurane inhalation. Following a midline abdominal incision, an atraumatic microvascular clamp was used to occlude the left kidney for 25–37 min. After the release of the clamp, a contralateral nefrectomy was performed.

Kidney functional measurements

Blood samples were collected by retro-orbital puncture. Serum urea and creatinine levels were measured using QuantiChrom assay kits based on the improved Jung and Jaffe methods, respectively (DIUR-500 and DICT-500, Gentaur, Brussels, Belgium) according to the manufacturer's instructions, or were determined using an ELAN analyzer (Eppendorf Merck, Hamburg, Germany) with Ecoline S + reagents (Diagnostic Systems GmbH, Holzheim, Germany) according to the manufacturer's instructions.

Histology

Kidneys were harvested, bisected longitudinally, fixed for 24 h in formalin and embedded in paraffin. Three-micrometer sections were stained with hematoxylin and eosin, modified Jones staining or periodic acid Schiff (PAS). Immunohistochemistry was performed on deparaffinzed sections following antigen retrieval in boiling 10 mM sodium citrate.

mRNA expression analysis

Total RNA was extracted from frozen kidney using TRIzol reagent (Invitrogen) and oligodT or hexamer-primed cDNA synthesized using SuperScript II (Invitrogen) according to the manufacturer's instructions. Quantitative real-time PCR was performed using an Opticon2 DNA Engine (MJ Research) or a MyIQ (Bio-Rad) with SYBR Green incorporation. Each sample was tested in duplo at least two times.

Glucose tolerance and insulin sensitivity tests

Mice were fasted overnight prior to testing. Following baseline blood glucose determination from tail blood of conscious, restrained mice, animals were injected with a bolus of glucose (1.5 mg glucose/gram body weight) or insulin (0.75 U kg^{-1} body weight) into the intraperitoneal cavity. Blood glucose determinations were performed as above at the indicated times following injection using a HemoCue glucose 201 RT blood glucose analyzer (HemoCue, Ängelholm, Sweden).

Statistics

Data are expressed as the mean ± SEM. Statistical analyses of data on urea, creatinine, histomorphology, immunohistochemistry and immunoblot was performed using Student's t-test. Survival was analysed by Kaplan-Meier (SPSSv11).

ACKNOWLEDGEMENTS

This research was supported in part by the Netherlands Organization for Health Research and Development, Research Institute for Diseases of the Elderly (60-60400-98-004), the National Institutes of Health (1PO1 AG17242-02), the Association for International Cancer Research (05-280) and the European Commission RISC-RAD (FI6R-CT-e2003-508842). JRM was a fellow of the Damon Runyon Cancer Research Fund (DRG 1677). MV was supported by a grant from the Dutch Kidney Foundation (C04.2105).

REFERENCES

1. HARMAN, D., AGING: A THEORY BASED ON FREE RADICAL AND RADIATION CHEMISTRY. J GERONTOL, 1956. 11(3): P. 298-300.
2. BECKMAN, K.B. AND B.N. AMES, THE FREE RADICAL THEORY OF AGING MATURES. PHYSIOL REV, 1998. 78(2): P. 547-81.
3. MARTIN, G.M. AND J. OSHIMA, LESSONS FROM HUMAN PROGEROID SYNDROMES. NATURE, 2000. 408(6809): P. 263-6.
4. BROOKS, P.J., ET AL., THE OXIDATIVE DNA LESION 8,5'-(S)-CYCLO-2'-DEOXYADENOSINE IS REPAIRED BY THE NUCLEOTIDE EXCISION REPAIR PATHWAY AND BLOCKS GENE EXPRESSION IN MAMMALIAN CELLS. J BIOL CHEM, 2000. 275(29): P. 22355-62.
5. HOEIJMAKERS, J.H., GENOME MAINTENANCE MECHANISMS FOR PREVENTING CANCER. NATURE, 2001. 411(6835): P. 366-74.
6. CITTERIO, E., ET AL., ATP-DEPENDENT CHROMATIN REMODELING BY THE COCKAYNE SYNDROME B DNA REPAIR-TRANSCRIPTION-COUPLING FACTOR. MOL CELL BIOL, 2000. 20(20): P. 7643-53.
7. GROISMAN, R., ET AL., THE UBIQUITIN LIGASE ACTIVITY IN THE DDB2 AND CSA COMPLEXES IS DIFFERENTIALLY REGULATED BY THE COP9 SIGNALOSOME IN RESPONSE TO DNA DAMAGE. CELL, 2003. 113(3): P. 357-67.
8. FOUSTERI, M., ET AL., COCKAYNE SYNDROME A AND B PROTEINS DIFFERENTIALLY REGULATE RECRUITMENT OF CHROMATIN REMODELING AND REPAIR FACTORS TO STALLED RNA POLYMERASE II IN VIVO. MOL CELL, 2006. 23(4): P. 471-82.
9. NANCE, M.A. AND S.A. BERRY, COCKAYNE SYNDROME: REVIEW OF 140 CASES. AM J MED GENET, 1992. 42(1): P. 68-84.
10. DOLLE, M.E., ET AL., INCREASED GENOMIC INSTABILITY IS NOT A PREREQUISITE FOR SHORTENED LIFESPAN IN DNA REPAIR DEFICIENT MICE. MUTAT RES, 2006. 596(1-2): P. 22-35.
11. VAN DER HORST, G.T., ET AL., UVB RADIATION-INDUCED CANCER PREDISPOSITION IN COCKAYNE SYNDROME GROUP A (CSA) MUTANT MICE. DNA REPAIR (AMST), 2002. 1(2): P. 143-57.
12. VAN DER HORST, G.T., ET AL., DEFECTIVE TRANSCRIPTION-COUPLED REPAIR IN COCKAYNE SYNDROME B MICE IS ASSOCIATED WITH SKIN CANCER PREDISPOSITION. CELL, 1997. 89(3): P. 425-35.
13. GORGELS, T.G., ET AL., RETINAL DEGENERATION AND IONIZING RADIATION HYPERSENSITIVITY IN A MOUSE MODEL FOR COCKAYNE SYNDROME. MOL CELL BIOL, 2007. 27(4): P. 1433-41.
14. NIEDERNHOFER, L.J., ET AL., A NEW PROGEROID SYNDROME REVEALS THAT GENOTOXIC STRESS SUPPRESSES THE SOMATOTROPH AXIS. NATURE, 2006. 444(7122): P. 1038-43.
15. SCHUMACHER, B., ET AL., DELAYED AND ACCELERATED AGING SHARE COMMON LONGEVITY ASSURANCE MECHANISMS. PLOS GENET, 2008. 4(8): P. E1000161.
16. VAN DE VEN, M., ET AL., ADAPTIVE STRESS RESPONSE IN SEGMENTAL PROGERIA RESEMBLES LONG-LIVED DWARFISM AND CALORIE RESTRICTION IN MICE. PLOS GENET, 2006. 2(12): P. E192.
17. VAN DER PLUIJM, I., ET AL., IMPAIRED GENOME MAINTENANCE SUPPRESSES THE GROWTH HORMONE--INSULIN-LIKE GROWTH FACTOR 1 AXIS IN MICE WITH COCKAYNE SYNDROME. PLOS BIOL, 2007. 5(1): P. E2.
18. WIJNHOVEN, S.W., ET AL., ACCELERATED AGING PATHOLOGY IN AD LIBITUM FED XPD(TTD) MICE IS ACCOMPANIED BY FEATURES SUGGESTIVE OF CALORIC RESTRICTION. DNA REPAIR (AMST), 2005. 4(11): P. 1314-24.
19. BARTKE, A. AND H. BROWN-BORG, LIFE EXTENSION IN THE DWARF MOUSE. CURR TOP DEV BIOL, 2004. 63: P. 189-225.
20. VAN DE VEN, M., ET AL., EXTENDED LONGEVITY MECHANISMS IN SHORT-LIVED PROGEROID MICE: IDENTIFICATION OF A PRESERVATIVE STRESS RESPONSE ASSOCIATED WITH SUCCESSFUL AGING. MECH AGEING DEV, 2007. 128(1): P. 58-63.
21. KELLY, K.J., DISTANT EFFECTS OF EXPERIMENTAL RENAL ISCHEMIA/REPERFUSION INJURY. J AM SOC NEPHROL, 2003. 14(6): P. 1549-58.
22. FRIEDEWALD, J.J. AND H. RABB, INFLAMMATORY CELLS IN ISCHEMIC ACUTE RENAL FAILURE. KIDNEY INT, 2004. 66(2): P. 486-91.
23. PADANILAM, B.J., CELL DEATH INDUCED BY ACUTE RENAL INJURY: A PERSPECTIVE ON THE CONTRIBUTIONS OF APOPTOSIS AND NECROSIS. AM J PHYSIOL RENAL PHYSIOL, 2003. 284(4): P. F608-27.
24. MEGYESI, J., ET AL., COORDINATION OF THE CELL CYCLE IS AN IMPORTANT DETERMINANT OF THE SYNDROME OF ACUTE RENAL FAILURE. AM J PHYSIOL RENAL PHYSIOL, 2002. 283(4): P. F810-6.
25. DEVARAJAN, P., CELLULAR AND MOLECULAR DERANGEMENTS IN ACUTE TUBULAR NECROSIS. CURR OPIN PEDIATR, 2005. 17(2): P. 193-9.

26. TAKADA, M., ET AL., THE CYTOKINE-ADHESION MOLECULE CASCADE IN ISCHEMIA/REPERFUSION INJURY OF THE RAT KIDNEY. INHIBITION BY A SOLUBLE P-SELECTIN LIGAND. J CLIN INVEST, 1997. 99(11): P. 2682-90.
27. KHER, A., ET AL., CELLULAR AND MOLECULAR MECHANISMS OF SEX DIFFERENCES IN RENAL ISCHEMIA REPERFUSION INJURY. CARDIOVASC RES, 2005. 67(4): P. 594-603.
28. DE WAARD, H., ET AL., CELL TYPE-SPECIFIC HYPERSENSITIVITY TO OXIDATIVE DAMAGE IN CSB AND XPA MICE. DNA REPAIR (AMST), 2003. 2(1): P. 13-25.
29. DE WAARD, H., ET AL., DIFFERENT EFFECTS OF CSA AND CSB DEFICIENCY ON SENSITIVITY TO OXIDATIVE DNA DAMAGE. MOL CELL BIOL, 2004. 24(18): P. 7941-8.
30. TANG, B.L., SIRT1, NEURONAL CELL SURVIVAL AND THE INSULIN/IGF-1 AGING PARADOX. NEUROBIOL AGING, 2006. 27(3): P. 501-5.
31. CHANDRASEKAR, B., ET AL., CALORIE RESTRICTION ATTENUATES INFLAMMATORY RESPONSES TO MYOCARDIAL ISCHEMIA REPERFUSION INJURY. AM J PHYSIOL HEART CIRC PHYSIOL, 2001. 280(5): P. H2094-102.
32. YU, Z.F. AND M.P. MATTSON, DIETARY RESTRICTION AND 2-DEOXYGLUCOSE ADMINISTRATION REDUCE FOCAL ISCHEMIC BRAIN DAMAGE AND IMPROVE BEHAVIORAL OUTCOME: EVIDENCE FOR A PRECONDITIONING MECHANISM. J NEUROSCI RES, 1999. 57(6): P. 830-9.
33. MATTSON, M.P., HORMESIS DEFINED. AGEING RES REV, 2008. 7(1): P. 1-7.
34. MURRY, C.E., R.B. JENNINGS, AND K.A. REIMER, PRECONDITIONING WITH ISCHEMIA: A DELAY OF LETHAL CELL INJURY IN ISCHEMIC MYOCARDIUM. CIRCULATION, 1986. 74(5): P. 1124-36.
35. SINCLAIR, D.A., TOWARD A UNIFIED THEORY OF CALORIC RESTRICTION AND LONGEVITY REGULATION. MECH AGEING DEV, 2005. 126(9): P. 987-1002.
36. TURTURRO, A., B.S. HASS, AND R.W. HART, DOES CALORIC RESTRICTION INDUCE HORMESIS? HUM EXP TOXICOL, 2000. 19(6): P. 320-9.
37. ARUMUGAM, T.V., ET AL., HORMESIS/PRECONDITIONING MECHANISMS, THE NERVOUS SYSTEM AND AGING. AGEING RES REV, 2006. 5(2): P. 165-78.

Chapter 4

Long-term results after renal ischemia reperfusion injury in DNA repair deficient mice

D Susa, JR Mitchell[#], HP Roest, S van den Engel, IM Bajema[‡], K Mangundap, JNM IJzermans, JHJ Hoeijmakers[#], RWF de Bruin

Department of Surgery, Department of Genetics[#], Erasmus Medical Center, Rotterdam, The Netherlands
Department of Pathology[‡], Leiden University Medical Center, Leiden, The Netherlands

ABSTRACT

Previously, we have reported that congenital DNA repair deficiency can result in activation of systemic protective mechanisms resulting in a reduced inflammatory response in the short-term. The question remains whether oxidative stress would in the long-term lead to functional or histological signs of accelerated renal ageing in DNA repair deficient mice.

Therefore, in a cohort of mice with deficiencies in NER proteins Csb, Ttd and Xpa were subjected to renal ischemia reperfusion. Functional and histologic data was collected over time.

Up to eleven months after ischemia reperfusion injury no difference was found in survival, histology, or kidney function as measured by creatinine, blood urea nitrogen (BUN), albuminuria and glomerular filtration rate (GFR) between NER-deficient mice and wild-type.

These data are in line with the assumption that unrepaired endogenous DNA lesions may elicit an adaptive response, which protects against acute oxidative injury, with sustained functional outcome in the long-term. Further elucidation of this adaptive response could lead to specific interventions to preserve kidney function on the short- and long-term after ischemic injury.

INTRODUCTION

Renal transplantation has, since the first successful transplantation in 1954 of a healthy kidney, become an increasingly successful medical treatment for patients with end-stage failure of various organs. However, kidney transplantation has still not achieved its full potential as a long-term treatment for end-stage organ failure. CTD is the single most important cause of late graft loss. Histologic signs of CTD include intimal hyperplasia of graft arteries, glomerular sclerosis, tubular atrophy and interstitial fibrosis, which overlap with the histology of normally aging kidneys.

Increasing evidence suggests that the development if CTD is multifactorial in origin, with allo-antigen dependent and allo-antigen independent factors contributing to the decline of renal graft function [1, 2]. Of the allo-antigen independent factors, donor age and cold ischemia time seem to be the major correlate to transplant survival [3]. Clinical observations suggest an inverse relationship between both donor age and ischemia time and long-term graft survival [4, 5]. The mechanism by which donor age or ischemia lead to reduced functional life span is currently unknown.

Reactive oxygen species (ROS) produced by normal cellular metabolism and in excess during ischemia reperfusion after transplantation are a primary source of cellular and DNA damage. Since the free radical theory as originally proposed by Harman [6], evidence for a molecular connection between oxidative DNA damage and ageing, aging-related disorders and so-called 'premature aging' disorders (here referred to as segmental progerias) is compelling, which suggests that the mechanism causing normal ageing and long-term organ rejection may fundamentally be the same.

To counteract oxidative DNA damage nature has evolved several efficient, highly conserved DNA repair mechanisms [7]. Nucleotide excision DNA repair (NER) is one such repair system that removes a range of damages [8]. Two basic modes of lesion recognition have been defined. In the first, lesions occurring anywhere in the genome are recognized by damage-binding proteins such as the XPC-HR23A/B-CEN1 complex. This triggers the assembly of the multiprotein NER machinery, which functions via a cut and patch mechanism to remove the damage and fill the remaining single-strand gap. This system is referred to as global-genome (GG)-NER. In the second mode of recognition, transcription-coupled (TC)-NER, lesions that block an elongating RNA polymerase trigger assembly of the NER machinery in a process that depends on the chromatin remodelling protein CSB [9] and a ubiquitin ligase complex containing the TC-NER specific protein CSA [10, 11]. Deficiencies in any of these proteins give rise to at least three distinct heritable human disorders: xeroderma pigmentosum (XP), trichothiodystrophy (TTD) and Cockayne syndrome (CS) [12-14]. Patients with XP exhibit a >1000-fold increase in UV-induced skin cancer, accelerated photo aging of the skin and frequent neurodegeneration [15]. XP can be classified by mutation in one of seven genes (XPA to XPG). Like human patients, Xpa mutant mice show an similar increased incidence of skin cancer [16]. In CS patients physical and neurological development are severely impaired, resulting in dwarphism, cachexia, loss of retinal cells and dysmyelination [17]. The reported average age of death is close to 12 years [18]. Mouse models lacking Csa or Csb function recapitulate some progeroid characteristics of the human disease, including cachexia and progressive loss of photoreceptor cells, but with a normal lifespan and an overall milder phenotype than in humans [19-21].

Whereas CS is caused by a defect only in TCR, the molecular basis of TTD is found in partial defects in GG-NER and TC-NER. This disorder arises from point mutations in the XPB, XPD and TTDA genes. Mutations in this gene can give rise to XP, CS or TTD. This is explained by the fact that XPD encodes for one of the two subunits of transcription factor II helicase (TFIIH), which is involved in both global genome and transcription repair, as well as in basal transcription initiation [22, 23]. TTD patients, affected by prominent symptoms of accelerated ageing, suffer from skeletal and neurological degeneration, cachexia, ichthyosis and characteristic brittle hair, nails and scaly skin. TTD patients have a reduced life expectancy, but extensive clinical heterogeneity exists, ranging from mild growth retardation to life-threatening cachexia. The Ttd mouse model closely resembles the human disorder. Ttd mice show reduced life span, osteoporosis, osteosclerosis, grey hair and cachexia [24, 25].

In support of the role of oxidative stress hypersensitivity in DNA repair deficiency syndromes, an increase in skin and corneal malignancies and loss of photoreceptor cells following whole-body ionizing radiation was observed in respectively *Ttd*$^{-/-}$, *Xpa*$^{-/-}$ and *Csb*$^{-/-}$ mice [25-27]. Thus, at least particular cell types appear hypersensitive to the effects of oxidative DNA damage, a property that may underlie tissue-specific disease phenotypes.

However, a number of recent reports have described that mouse models of NER-deficient segmental progerias (NER progerias) display characteristics of hypopituitary dwarf mutants or dietary restricted wild type mice [28-32]. These phenotypes, including reduced body weight and temperature, hypoglycemia, hypoinsulinemia and reduced serum IGF-1, are correlated in dwarf and dietary restricted mice not only with extended longevity but also resistance to oxidative stress [33].

In the present study, we wondered whether DNA repair deficient mouse models exposed to acute renal oxidative stress, would show an increased stress resistance, as seen in models of dwarf-, and dietary restricted mice [33], or would be hypersensitive and show long-term deterioration of kidney function triggered by the acute insult, as seen in photoreceptor cells following whole-body ionizing radiation.

EXPERIMENTAL PROCEDURES

Animals

The experimental protocol was approved by the Animal Experiment Committee under the national Experiments on Animals Act and adhered to the rules laid down in this national law that serves the implementation of "Guidelines on the protection of experimental animals" by the Council of Europe (1986), Directive 86/609/EC. Male *Csb*$^{-/-}$ (n=9) [21], *Xpa*$^{-/-}$ (n=4) [35] and *Ttd*$^{-/-}$ (n=9) [36] mice in a C57BL/6 background were bred at the animal facility of the Erasmus Medical Centre; C57BL/6 J mice were purchased from Harlan, Horst, the Netherlands and were assigned to the sham group (n=5) or the experimental group (n=8).

Ischemia model

Mice between 12 and 16 weeks of age were anaesthetized by isoflurane inhalation. Following a midline abdominal incision, an atraumatic microvascular clamp was used to occlude the left kidney for 30 min. After the release of the clamp, a contralateral nefrectomy was performed. Animals were sacrificed 11 months after reperfusion.

Kidney functional measurements

Blood samples were collected by retro-orbital puncture. Serum urea and creatinine values were measured using a kinetic urease method where the decrease in NADH adsorbance is measured photometrically, using an ELAN multianalyser (Eppendorf-Merck, Germany). Urine was collected monthly by placing the mice individually in metabolic cages (Tecniplast, Buguggiate (Va), Italy) for 24 hours. Protein excretion was measured colorimetrically by addition of red [37]. The glomerular filtration rate (GFR) per 100 g body weight (BW) was based on the clearance of creatinine (GFR (ml/min) = $[\text{creatinine}]_{urine}$ multiplied by 24h volume/ $[\text{creatinine}]_{serum}$).

Histology

Kidneys were harvested, bisected longitudinally, fixed for 24 h in formalin and embedded in paraffin. Three-micrometer sections were stained with hematoxylin and eosin, modified Jones staining or periodic acid Schiff (PAS) and assessed by light microscopy for presence of proteincontaining tubules, microcalcifications, cell death and regeneration, in a blind fashion by I.M. Bajema.

Statistics

Statistical analysis was evaluated by one-way analysis of variance with Bonferroni's post hoc test. $p<0.05$ was taken to denote statistical significance. Data are expressed as mean±SEM (SPSSv11).

RESULTS

Weight

During the experiment there was an increase in the weight of the mice in the sham, wild-type and XPA group (Figure 1). In the CSB and TTD group the weight of the mice remained stable. At the end of the study period there was a significant difference in weight of the control wild-type compared to the CSB and TTD groups.

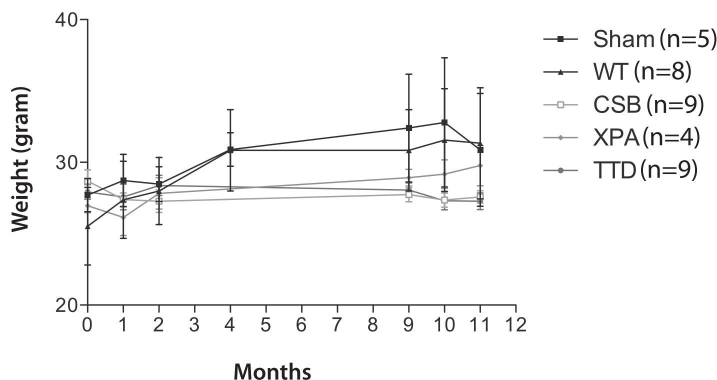

Figure 1. During the experiment there was an increase in the weight of the mice in the sham, wild-type and XPA group. In the CSB and TTD group the weight of the mice remained stable. At the end of the study period there was a significant difference in weight of the control wild-type compared to the CSB and TTD groups.

Renal function

In total seven animals died during the 11 month study period. One mouse in the sham group died after eleven months, one mouse in the wild-type nephrectomized group also after 11 months, two mice in the CSB group, one in the second month and the other after eleven months, 2 mice in the TTD group after eight months and one mouse in the XPA group after 11 months. In all cases, mortality was not associated with signs of renal dysfunction, such as an increase in mean blood urea nitrogen (BUN) or proteinuria. At autopsy, all kidneys had a normal appearance. Figure 2A-D shows the results of serum creatinine, BUN, albuminuria, GFR in mice after 30 minutes of warm ischemia of the left kidney. There were no significant differences in mean serum creatinine between the groups during the experiment.

Mean BUN compared between the sham, wild-type, CSB and XPA group did not significantly differ over time. However, mean BUN in the TTD group was significantly elevated during the experiment. Also, a rise in BUN was seen after 11 months, compared to BUN before clamping in the CSB, XPA and TTD groups. This elevated BUN after 11 months was not seen in the control wild-type and sham operated group.

Urinary albumine excretion did not statistically differ in all the groups and was stable throughout the experiment.

At the first month, GFR was higher in the TTD group compared to the XPA group, however no significant difference was found when compared to the control wild-type group. Except for this timepoint, no statistical difference was found between the groups and the GFR remained stable.

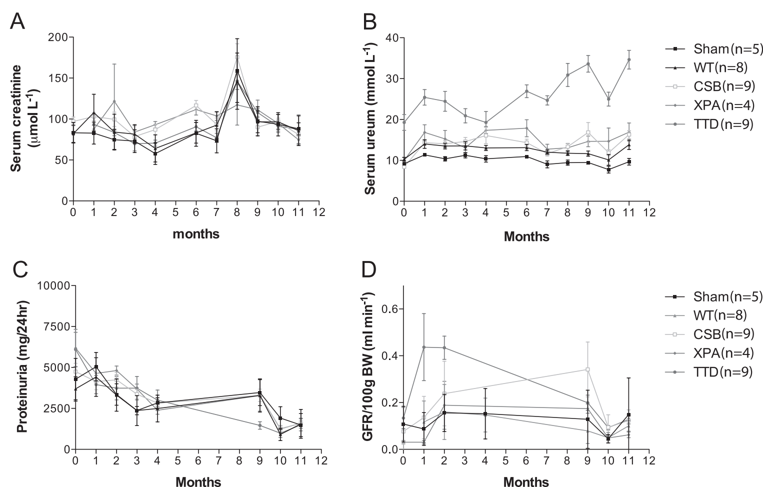

Figure 2. (A) There were no significant differences in mean serum creatinine between the groups during the experiment. (B) Mean BUN compared between the sham, wild-type, CSB and XPA group did not significantly differ over time. However mean BUN in the TTD group was significantly elevated during the experiment. Also, a rise in BUN was seen after 11 months, compared to BUN before clamping in the CSB, XPA and TTD groups. This elevated BUN after 11 months was not seen in the control wild-type and sham operated group. (C) At the first month, GFR was higher in the TTD group compared to the XPA group, however no significant difference was found when compared to the control wild-type group. Except for this timepoint, no statistical difference was found between the groups and the GFR remained stable. (D) There were no significant differences in mean proteinuria between the groups during the experiment

Macroscopic appearance and histology

At the end of the experiment, the remaining kidney of all groups had a normal macroscopic appearance. Histomorphologically, no glomerular changes were seen. Protein containing tubules, microcalcifications, cell-death and regeneration were not seen. Minor focal interstitial infiltration was noted in 2 animals in the XPA group, in 3 animals in the CSB group and 1 in the control wild type group.

DISCUSSION

CTD is the single most important cause of late graft deterioration and failure. Clinical observations suggest an inverse relationship between ischemia time and long-term graft survival [5] and have led to the assumption that ischemic injury leads to premature ageing of organs and reduced lifespan [38, 39]. The mechanism by which ischemia leads to reduced lifespan is currently unknown, but clearly is not solely dependent of the host alloimmune response [40, 41]. To explore the relationship between renal ischemia reperfusion injury, long-term renal dysfunction and premature ageing, we studied the effects of long-term renal ischemia reperfusion injury in mice with premature aging syndromes due to specific GG-NER and TC-NER deficiencies. A follow-up period of 11 months was based on results from previous experimental studies, in which clamping of renal vessels in rats led to renal dysfunction from 8 weeks post ischemia reperfusion injury [42], and on previous observations that these mice have a reduced lifespan [7].

The use of a clamping model enabled us to study the influence of ischemia reperfusion injury as an isolated factor on renal function and morphology, in the absence of an allogeneic response.

The present study demonstrates that up to 11 months an ischemic insult of 30 minutes in unilaterally nephrectomized wild-type mice does not lead to long-term changes in renal function nor histological changes, as observed in CAN. Clamping of the renal vascular pedicle did result in short-term deterioration of renal function in all groups (data not shown), but in all groups a subsequent recovery was observed. Most experimental studies evaluating renal I/R injury in mice have focused only on acute events after ischemia. Long-term outcome has been subject of only a few studies [43, 44]. Burne-Taney et al. applied 60 minutes of warm unilateral ischemia without contralateral nephrectomy. They report a fibrotic kidney 6 weeks after ischemia. Sixty minutes of ischemia was chosen because it produces severe damage to the kidney. Therefore it is quite likely that the ischemic kidney did not recover from the injury at all, and the animals survived on their remaining undamaged kidney. This makes interpretation of these data difficult. In our model 30 minutes of unilateral ischemia was followed by contralateral nephrectomy, leaving the animal dependent on its damaged kidney. Longer ischemia times in our hands resulted in high mortality rates due to renal dysfunction (data not shown). Only when kidneys were transplanted over a MHC barrier long-term changes as seen in CAN, could be detected . In contrast in rat models, IRI led to histological and functional changes 8 weeks after clamping [40]. Collectively, these data strongly suggest that in mouse models of both alloantigen-dependent and –independent factors are needed to produce CAN-like features.

Like in the wild-type animals, in the DNA repair deficient mice no significant difference in renal function or morphology was observed in 11 months. A significant difference was found in serum

urea concentration in the TTD mice, but this did not reflect renal function, as serum creatinine levels were not elevated and no morphologic abnormalities could be detected. The elevated serum urea levels could point to dehydration in these mice. In hypovolemic states, an increase in proximal tubule sodium reabsorption is seen [45]. This produces a parallel increase in urea reabsorption, as urea aborption is passively linked to reaborption of sodium and water. The net effect is a fall in urea excretion and elevations in the serum urea concentration. On the other hand serum creatinine concentration does not rise under these conditions. In normal subjects the serum urea/creatinine ratio is approximately 10:1, however in hypovolemic states this value frequently rises to greater than 20:1 [46]. In TTD mice we found a serum urea/creatinine ratio of 30:1. Hypovolemia in these mice fits in the phenotype of premature aging, as an impaired thirst mechanism is frequently seen in elderly subjects [47]. In our study, after 11 months follow-up, CSB and TTD mice weighted significantly less than wild-type and XPA mice. This is in line with the cachectic features in mice with CS and TTD features.

Although accelerated aging has been put forward to contribute to the pathophysiology of CTD [38, 39, 48], we could not demonstrate the involvement of NER pathways. On the contrary, recently we have reported increased resistance to acute oxidative stress in kidneys of *Csb*$^{-/-}$ mice after renal ischemia reperfusion injury on short-term function [34]. This congenital DNA repair deficiency seems to result in the activation of a systemic protective mechanism protecting against acute oxidative injury. The results in the present study underscore these findings. The protection against the acute consequences of renal ischemia reperfusion injury in these mice predicts that the long-term sequelae of this injury are also absent.

We wondered whether DNA repair deficient mouse models exposed to renal oxidative stress, would show an increased stress resistance, as seen in models of dwarf-, and dietary restricted mice, or would be hypersensistive, as seen in photoreceptor loss after whole body ionizing radiation. Here we report that acute oxidative stress does not lead to alteration of long-term term function, histology nor survival in mouse deficient of Xpa, Csb and Ttd proteins. Although the exact mechanism still has to be revealed, we propose that unrepaired endogenous DNA lesions elicit an adaptive hormetic response, which protects against acute oxidative injury, with sustained functional outcome in the long-term. Further elucidation of these adaptive responses could lead to specific interventions to preserve kidney function after oxidative injury on short- and long-term.

REFERENCES

1. KOUWENHOVEN, E.A., I.J. JN, AND R.W. DE BRUIN, ETIOLOGY AND PATHOPHYSIOLOGY OF CHRONIC TRANSPLANT DYSFUNCTION. TRANSPL INT, 2000. 13(6): P. 385-401.
2. HARIHARAN, S., LONG-TERM KIDNEY TRANSPLANT SURVIVAL. AM J KIDNEY DIS, 2001. 38(6 SUPPL 6): P. S44-50.
3. TERASAKI, P.I., J.M. CECKA, AND D.W. GJERTSON, IMPACT ANALYSIS: A METHOD FOR EVALUATING THE IMPACT OF FACTORS IN CLINICAL RENAL TRANSPLANTATION. CLIN TRANSPL, 1998: P. 437-41.
4. MORRIS, P.J., ET AL., ANALYSIS OF FACTORS THAT AFFECT OUTCOME OF PRIMARY CADAVERIC RENAL TRANSPLANTATION IN THE UK. HLA TASK FORCE OF THE KIDNEY ADVISORY GROUP OF THE UNITED KINGDOM TRANSPLANT SUPPORT SERVICE AUTHORITY (UKTSSA). LANCET, 1999. 354(9185): P. 1147-52.
5. SHOSKES, D.A. AND J.M. CECKA, DELETERIOUS EFFECTS OF DELAYED GRAFT FUNCTION IN CADAVERIC RENAL TRANSPLANT RECIPIENTS INDEPENDENT OF ACUTE REJECTION. TRANSPLANTATION, 1998. 66(12): P. 1697-701.
6. HARMAN, D., AGING: A THEORY BASED ON FREE RADICAL AND RADIATION CHEMISTRY. J GERONTOL, 1956. 11(3): P. 298-300.
7. HOEIJMAKERS, J.H., GENOME MAINTENANCE MECHANISMS FOR PREVENTING CANCER. NATURE, 2001. 411(6835): P. 366-74.
8. BROOKS, P.J., ET AL., THE OXIDATIVE DNA LESION 8,5'-(S)-CYCLO-2'-DEOXYADENOSINE IS REPAIRED BY THE NUCLEOTIDE EXCISION REPAIR PATHWAY AND BLOCKS GENE EXPRESSION IN MAMMALIAN CELLS. J BIOL CHEM, 2000. 275(29): P. 22355-62.
9. CITTERIO, E., ET AL., ATP-DEPENDENT CHROMATIN REMODELING BY THE COCKAYNE SYNDROME B DNA REPAIR-TRANSCRIPTION-COUPLING FACTOR. MOL CELL BIOL, 2000. 20(20): P. 7643-53.
10. FOUSTERI, M., ET AL., COCKAYNE SYNDROME A AND B PROTEINS DIFFERENTIALLY REGULATE RECRUITMENT OF CHROMATIN REMODELING AND REPAIR FACTORS TO STALLED RNA POLYMERASE II IN VIVO. MOL CELL, 2006. 23(4): P. 471-82.
11. GROISMAN, R., ET AL., THE UBIQUITIN LIGASE ACTIVITY IN THE DDB2 AND CSA COMPLEXES IS DIFFERENTIALLY REGULATED BY THE COP9 SIGNALOSOME IN RESPONSE TO DNA DAMAGE. CELL, 2003. 113(3): P. 357-67.
12. BOOTSMA, D., KRAEMER, K. H., CLEAVER, J. & HOEIJMAKERS, J. H. J., IN THE METABOLIC AND MOLECULAR BASIS OF INHERITED DISEASE VOL. 1 (EDS SCRIVER, C. R., BEAUDET, A. L., SLY, W. S. & VALLE, D.) 677-703 (MCGRAW-HILL, NEW YORK, 2001). 2001.
13. LEHMANN, A.R., THE XERODERMA PIGMENTOSUM GROUP D (XPD) GENE: ONE GENE, TWO FUNCTIONS, THREE DISEASES. GENES DEV, 2001. 15(1): P. 15-23.
14. DE BOER, J. AND J.H. HOEIJMAKERS, CANCER FROM THE OUTSIDE, AGING FROM THE INSIDE: MOUSE MODELS TO STUDY THE CONSEQUENCES OF DEFECTIVE NUCLEOTIDE EXCISION REPAIR. BIOCHIMIE, 1999. 81(1-2): P. 127-37.
15. HASTY, P., ET AL., AGING AND GENOME MAINTENANCE: LESSONS FROM THE MOUSE? SCIENCE, 2003. 299(5611): P. 1355-9.
16. DE LAAT, W.L., N.G. JASPERS, AND J.H. HOEIJMAKERS, MOLECULAR MECHANISM OF NUCLEOTIDE EXCISION REPAIR. GENES DEV, 1999. 13(7): P. 768-85.
17. VAN GOOL, A.J., ET AL., COCKAYNE SYNDROME: DEFECTIVE REPAIR OF TRANSCRIPTION? EMBO J, 1997. 16(14): P. 4155-62.
18. NANCE, M.A. AND S.A. BERRY, COCKAYNE SYNDROME: REVIEW OF 140 CASES. AM J MED GENET, 1992. 42(1): P. 68-84.
19. DOLLE, M.E., ET AL., INCREASED GENOMIC INSTABILITY IS NOT A PREREQUISITE FOR SHORTENED LIFESPAN IN DNA REPAIR DEFICIENT MICE. MUTAT RES, 2006. 596(1-2): P. 22-35.
20. VAN DER HORST, G.T., ET AL., UVB RADIATION-INDUCED CANCER PREDISPOSITION IN COCKAYNE SYNDROME GROUP A (CSA) MUTANT MICE. DNA REPAIR (AMST), 2002. 1(2): P. 143-57.
21. VAN DER HORST, G.T., ET AL., DEFECTIVE TRANSCRIPTION-COUPLED REPAIR IN COCKAYNE SYNDROME B MICE IS ASSOCIATED WITH SKIN CANCER PREDISPOSITION. CELL, 1997. 89(3): P. 425-35.
22. VERMEULEN, W., ET AL., A TEMPERATURE-SENSITIVE DISORDER IN BASAL TRANSCRIPTION AND DNA REPAIR IN HUMANS. NAT GENET, 2001. 27(3): P. 299-303.
23. LITHGOW, G.J. AND M.S. GILL, PHYSIOLOGY: COST-FREE LONGEVITY IN MICE? NATURE, 2003. 421(6919): P. 125-6.
24. DE BOER, J., ET AL., PREMATURE AGING IN MICE DEFICIENT IN DNA REPAIR AND TRANSCRIPTION. SCIENCE, 2002. 296(5571): P. 1276-9.
25. DE BOER, J., ET AL., MOUSE MODEL FOR THE DNA REPAIR/BASAL TRANSCRIPTION DISORDER TRICHOTHIODYSTROPHY REVEALS CANCER PREDISPOSITION. CANCER RES, 1999. 59(14): P. 3489-94.

26. DE VRIES, A., ET AL., ULTRAVIOLET-B INDUCED HYPERPLASIA AND SQUAMOUS CELL CARCINOMAS IN THE CORNEA OF XPA-DEFICIENT MICE. EXP EYE RES, 1998. 67(1): P. 53-9.
27. GORGELS, T.G., ET AL., RETINAL DEGENERATION AND IONIZING RADIATION HYPERSENSITIVITY IN A MOUSE MODEL FOR COCKAYNE SYNDROME. MOL CELL BIOL, 2007. 27(4): P. 1433-41.
28. NIEDERNHOFER, L.J., ET AL., A NEW PROGEROID SYNDROME REVEALS THAT GENOTOXIC STRESS SUPPRESSES THE SOMATOTROPH AXIS. NATURE, 2006. 444(7122): P. 1038-43.
29. SCHUMACHER, B., ET AL., DELAYED AND ACCELERATED AGING SHARE COMMON LONGEVITY ASSURANCE MECHANISMS. PLOS GENET, 2008. 4(8): P. E1000161.
30. VAN DE VEN, M., ET AL., ADAPTIVE STRESS RESPONSE IN SEGMENTAL PROGERIA RESEMBLES LONG-LIVED DWARFISM AND CALORIE RESTRICTION IN MICE. PLOS GENET, 2006. 2(12): P. E192.
31. VAN DER PLUIJM, I., ET AL., IMPAIRED GENOME MAINTENANCE SUPPRESSES THE GROWTH HORMONE--INSULIN-LIKE GROWTH FACTOR 1 AXIS IN MICE WITH COCKAYNE SYNDROME. PLOS BIOL, 2007. 5(1): P. E2.
32. WIJNHOVEN, S.W., ET AL., ACCELERATED AGING PATHOLOGY IN AD LIBITUM FED XPD(TTD) MICE IS ACCOMPANIED BY FEATURES SUGGESTIVE OF CALORIC RESTRICTION. DNA REPAIR (AMST), 2005. 4(11): P. 1314-24.
33. BARTKE, A. AND H. BROWN-BORG, LIFE EXTENSION IN THE DWARF MOUSE. CURR TOP DEV BIOL, 2004. 63: P. 189-225.
34. SUSA, D., ET AL., CONGENITAL DNA REPAIR DEFICIENCY RESULTS IN PROTECTION AGAINST RENAL ISCHEMIA REPERFUSION INJURY IN MICE. AGING CELL, 2009. 8(2): P. 192-200.
35. DE VRIES, A., ET AL., INCREASED SUSCEPTIBILITY TO ULTRAVIOLET-B AND CARCINOGENS OF MICE LACKING THE DNA EXCISION REPAIR GENE XPA. NATURE, 1995. 377(6545): P. 169-73.
36. DE BOER, J., ET AL., A MOUSE MODEL FOR THE BASAL TRANSCRIPTION/DNA REPAIR SYNDROME TRICHOTHIODYSTROPHY. MOL CELL, 1998. 1(7): P. 981-90.
37. WATANABE, N., ET AL., URINARY PROTEIN AS MEASURED WITH A PYROGALLOL RED-MOLYBDATE COMPLEX, MANUALLY AND IN A HITACHI 726 AUTOMATED ANALYZER. CLIN CHEM, 1986. 32(8): P. 1551-4.
38. HALLORAN, P.F., A. MELK, AND C. BARTH, RETHINKING CHRONIC ALLOGRAFT NEPHROPATHY: THE CONCEPT OF ACCELERATED SENESCENCE. J AM SOC NEPHROL, 1999. 10(1): P. 167-81.
39. SUSA, D., MECHANISMS OF AGEING IN CHRONIC ALLOGRAFT NEPHROPATHY. JOURNAL OF ORGAN DYSFUNCTION 2007. 3 (1): P. 7-17.
40. AZUMA, H., ET AL., INITIAL ISCHEMIA/REPERFUSION INJURY INFLUENCES LATE FUNCTIONAL AND STRUCTURAL CHANGES IN THE KIDNEY. TRANSPLANT PROC, 1997. 29(1-2): P. 1528-9.
41. SALAHUDEEN, A.K., N. HAIDER, AND W. MAY, COLD ISCHEMIA AND THE REDUCED LONG-TERM SURVIVAL OF CADAVERIC RENAL ALLOGRAFTS. KIDNEY INT, 2004. 65(2): P. 713-8.
42. AZUMA, H., ET AL., CELLULAR AND MOLECULAR PREDICTORS OF CHRONIC RENAL DYSFUNCTION AFTER INITIAL ISCHEMIA/REPERFUSION INJURY OF A SINGLE KIDNEY. TRANSPLANTATION, 1997. 64(2): P. 190-7.
43. FEITOZA, C.Q., ET AL., INHIBITION OF COX 1 AND 2 PRIOR TO RENAL ISCHEMIA/REPERFUSION INJURY DECREASES THE DEVELOPMENT OF FIBROSIS. MOL MED, 2008. 14(11-12): P. 724-30.
44. BURNE-TANEY, M.J., N. YOKOTA, AND H. RABB, PERSISTENT RENAL AND EXTRARENAL IMMUNE CHANGES AFTER SEVERE ISCHEMIC INJURY. KIDNEY INT, 2005. 67(3): P. 1002-9.
45. MOLITORIS, B.A., ACUTE RENAL FAILURE. DRUGS TODAY (BARC), 1999. 35(9): P. 659-66.
46. SKLAR, A.H., ET AL., PRERENAL AZOTEMIA: DIFFERENTIATION OF HYPERUREAGENESIS FROM RENAL HYPOPERFUSION USING URINARY UREA NITROGEN DATA. INT J ARTIF ORGANS, 1996. 19(3): P. 164-9.
47. SILVER, A.J. AND J.E. MORLEY, ROLE OF THE OPIOID SYSTEM IN THE HYPODIPSIA ASSOCIATED WITH AGING. J AM GERIATR SOC, 1992. 40(6): P. 556-60.
48. CHKHOTUA, A., ET AL., REPLICATIVE SENESCENCE IN ORGAN TRANSPLANTATION-MECHANISMS AND SIGNIFICANCE. TRANSPL IMMUNOL, 2002. 9(2-4): P. 165-71.

Chapter 5

On the Role of Adenosine In Classical and Interorgan Preconditioning of the Heart

DA Liem, D Susa, A Heinen, WE Bos, PD Verdouw, DJ Duncker

Department of Experimental Cardiology, Thoraxcenter, Erasmus University Rotterdam, Rotterdam, The Netherlands

In: Myocardial Ischemia and Preconditioning, edited by NS Dhalla, N Takeda, M Singh, A Lukas, Chapter 22: 317 – 328. Kluwer Academic Publishers, Boston 2003

ABSTRACT

Although adenosine is cardioprotective in the rat, there is general agreement that adenosine is not involved in ischemic preconditioning (IPC) in this species. Because evidence is accumulating that the signal transduction pathway leading to cardioprotection by IPC is stimulus-dependent, we hypothesized that the failure to show a role tor adenosine might be due to the use of brief IPC stimuli. We therefore investigated whether myocardial adenosine receptor activation (and which of its subtypes) would play a role in IPC in the rat heart when longer IPC stimuli are employed. The concept of ischemic preconditioning (intraorgan protection) has been expanded to interorgan preconditioning (IOPC; myocardial protection by brief ischemia in other organs). The mechanism underlying IOPC remains elusive, however, although a role for adenosine and activation of a neurogenic pathway has been postulated. We therefore also tested the hypothesis that adenosine released by the remote ischemic organ stimulates local afferent nerves, which leads to activation of myocardial adenosine receptors.

In pentobarbital-anesthetized rats, a preconditioning stimulus consisting of 3 sequences of 3-min coronary artery occlusion (CAO) each followed by 5 min of reperfusion limited infarct size produced by a 60-min CAO which was not affected by a high dose of the adenosine receptor antagonist 8-(p-sulfophenyl)theophylline (8-SPT). In contrast, the protection afforded by a single 15-min CAO was abolished by the same dose of 8-SPT demonstrating not only the involvement of adenosine in IPC in rats, but also that this depends critically on the IPC stimulus. Selective blockade of A_1- (low dose of 8-SPT) or A_3-receptors (MRS-1191), blunted the protection by classical IPC with a 15-min CAO, while a combination of 10SPT and MRS 1191 abolished classical IPC, which implies that activation of both A_1- and A_3-receptors contributed to the protection by the 15-min CAO.

IOPC with a 15-min mesenteric artery occlusion (MAO15), reduced infarct size produced by a 60-min CAO, which was abolished by pretreatment with the ganglion blocker hexamethonium, but not when hexamethonium was administered at 5 min of mesenteric artery reperfusion. In contrast, 8-SPT abolished IOPC independent of the timing of administration (either before or 5 min following MAO15), indicating that adenosine receptors downstream of the neurogenic pathway (i.e. in the heart) are involved. Involvement of small intestinal adenosine receptors is suggested by the finding that intramesenteric artery (but not intraportal vein) infusion of adenosine (10 μg/min) was as cardioprotective as MAO15. This protection was also prevented by hexamethonium. In conclusion, in IOPC by a 15-min MAO adenosine locally released during small intestinal ischemia stimulates afferent nerves in the mesenteric bed during early reperfusion, thereby initiating a neurogenic pathway that ultimately leads to activation of myocardial adenosine receptors and hence cardioprotection.

INTRODUCTION

Based on numerous studies including those in which the selective adenosine A_1-receptor antagonist PD 115,199 and the non-selective adenosine antagonist 8-SPT failed to block ischemic preconditioning (IPC) [1-3], Ganote and Armstrong [4] concluded that myocardial adenosine receptor activation, in contrast to all other species studied, is not involved in IPC in the rat although administration of exogenous adenosine is cardioprotective in this species [5]. In the aforementioned IPC studies [1-3] the duration of the (multiple) IPC stimuli was 3-5 min. Interestingly, intracoronary adenosine deaminase is ineffective in attenuating infarct size limitation by a 3-min coronary artery occlusion (CAO), but abolishes the cardioprotection by a 10-min CAO in swine [6]. We therefore hypothesized that adenosine might also play a role in IPC in rats when preconditioning stimuli of longer duration are used. We therefore investigated the putative involvement of adenosine in IPC by a 15-min CAO, a stimulus which has been shown to protect the rat heart [7, 8]. We also preconditioned rat hearts by three cycles of 3-min CAO and 5 min of reperfusion (as used by Li and Kloner [2]), to exclude that differences in breed, sex and experimental procedures acted as confounding factors. Our results not only confirmed the findings by Li and Kloner [2] but also demonstrated that a high dose of 8-SPT completely blocked the cardioprotection by a single 15-min CAO stimulus. Since 8-SPT is a non-selective adenosine receptor antagonist and evidence is accumulating that not only selective A_1- but also selective A_3- agonists confer cardioprotection [9-11], we subsequently investigated whether both A_1- and A_3- the adenosine receptor subtypes are involved in IPC [12].

Przyklenk et al. [13] expanded the concept of ischemic preconditioning from intraregional to interregional myocardial protection by showing that a brief coronary artery occlusion (CAO) not only preconditioned the distribution area of that coronary artery but also protected the adjacent virgin myocardium. In our laboratory [7], we subsequently showed that 15 min of small intestinal or renal ischemia preceding a 60-min CAO by 10 min also limited myocardial infarct size. This interorgan preconditioning (IOPC; remote myocardial preconditioning; preconditioning at a distance) of the heart by preceding transient ischemia in remote organs has been confirmed for the small intestine [14, 15], kidney [16-18], and skeletal muscle [19], but may not apply to all remote organs as global cerebral ischemia preceding a 60-min CAO did not limit infarct size [20]. The mechanism underlying IOPC remains elusive, although Gho et al. [7] showed the involvement of a neurogenic pathway, as pretreatment with the ganglion blocker hexamethonium abolished IOPC by small intestinal ischemia. Reperfusion of the occluded mesenteric artery before the onset of the 60-min CAO was mandatory as reopening of the mesenteric artery within 1 min after the coronary artery was occluded did not confer cardioprotection. This finding suggests that activation of the neurogenic pathway occurs up on mesenteric artery reperfusion [7]. Adenosine may also be involved in IOPC [17, 18], but the site(s) of action of this mediator, i.e. the ischemic organ or the heart or both, was not investigated. Takaoka et al. [18] proposed that myocardial adenosine receptors might be involved because the adenosine concentrations in the carotid artery after renal ischemia exceeded those after regional myocardial ischemia. On the other hand, Pell et al. [17] concluded from the degree of hypotension following renal artery reperfusion, that adenos-

ine release from the kidney was insufficient to produce cardioprotection via the circulation. They suggested, based on the observations by Gho et al. [7], that adenosine released in the ischemic kidney stimulated the afferent renal nerves and thereby protected the myocardium, a hypothesis that is supported by recent experiments in anesthetized rabbits [16]. To further elucidate the mechanism of IOPC, we not only investigated whether activation of adenosine receptors is involved in IOPC by small intestinal ischemia, but we also determined the location(s) (myocardium and/or small intestine) of the adenosine receptors involved [21].

METHODS

Experimental procedures

Pentobarbital anesthetized (60mg/kg, ip) male Wistar rats (300g) were intubated (PE-240) for positive pressure ventilation (Harvard rodent ventilator) with room air [7, 8, 12, 21]. The thoracic aorta was cannulated via the carotid artery with a PE-50 catheter for measurement of arterial blood pressure and computation of heart rate. A catheter was positioned in the femoral vein for infusion of Haemacell (Behringwerke) to maintain fluid balance. After thoracotomy via the left third intercostal space and opening of the pericardium, a silk 6-0 suture was looped under the coronary artery for later occlusion. In animals that underwent IOPC, a laparotomy was performed and the anterior mesenteric artery was dissected free and looped by a loose suture to allow later mesenteric artery occlusion with an atraumatic clamp. Pentobarbital was suffused in the abdominal cavity to maintain anesthesia. Rectal temperature was continuously measured and was maintained at 36.5-37.5°C (8).

Rats that fibrillated during occlusion or reperfusion were allowed to complete the protocol when conversion to normal sinus rhythm occurred spontaneously within 1 min, or when resuscitation by gently thumping on the thorax or defibrillation with a modified battery of 9 V was successful within 2min after onset of fibrillation.

After surgery, a 30-min stabilization period was allowed before the start of the experimental protocol. All animals were subjected to a 60-min CAO followed by 120 min of reperfusion, after which area at risk (AR) and infarct area (IA) were determined using trypan blue and nitro-blue-tetrazolium staining [7, 8, 12, 21]. Infarct size (IS), defined as 100 x IA/AR (%), was analyzed by one-way analysis of variance followed by Student-Newman-Keuls test. Statistical significance was accepted when $P < 0.05$. Data are mean ± SEM.

RESULTS

Protocol I: Involvement of adenosine in classical ischemic preconditioning in the rat heart.

Figure 1 shows that classic al IPC by a single 15-min CAO followed by 10-min of reperfusion (CAO15), limited infarct size to 45 ± 2% versus 69 ± 1 % in Sham rats that underwent only the 60-min CAO. 8-SPT in a non-selective dose of 2 X 25 mg/kg (50SPT), had no effect on infarct size in Sham rats, but abolished the protection afforded by CAO15. The figure also shows that IPC by 3 cycles of 3-min CAO and 5 min of reperfusion (3-CAO3), limited infarct size to 24 ± 6%, but that 50SPT did not attenuate the cardioprotection (IS = 18 ± 3%), confirming earlier observations by Li and Kloner [2].

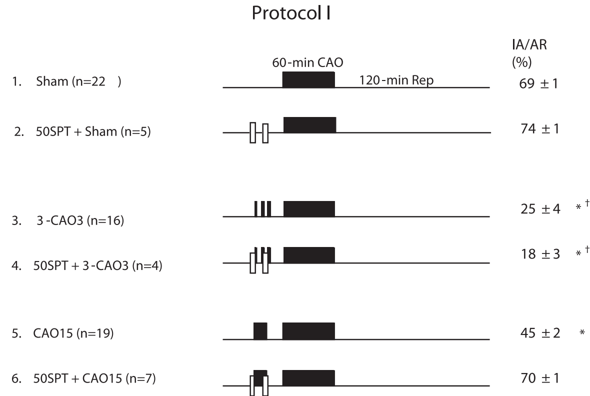

Figure 1. Both 3-CAO3 and CAO15 (although to a lesser extent) limit infarct size, but only the protection by CAO15 is abolished by 8-SPT. *P < 0.05 vs Sham; †P < 0.05 vs corresponding CAO15.

Protocol II: Involvement of adenosine A_1- and A_3-receptor subtypes in ischemic preconditioning in the rat heart.

Figure 2 shows that cardioprotection by IPC with CAO15 was blunted by 50% when rats were pretreated with either an A_1-receptor selective dose of 8-SPT (10SPT) [12] or an A_3-receptor selective dose of MRS-1191 [12]. Combination of 10SPT and MRS-1191 abolished the cardioprotection by CAO15 (10SPT + MRS + CAO15), indicating that both A_1- and A_3-receptors contributed to IPC in the rat heart.

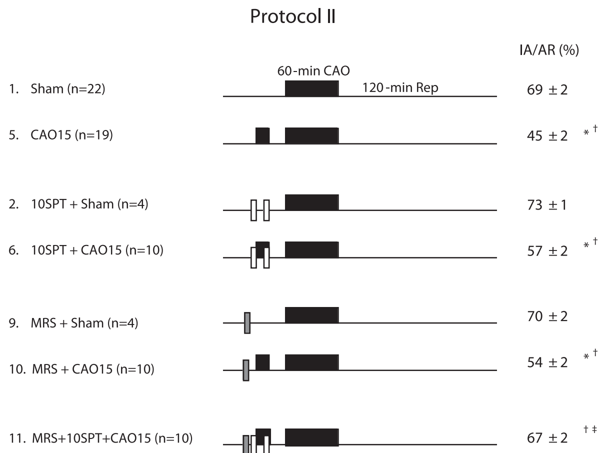

Figure 2. Infarct size limitation by CAO15 is attenuated by the A_1 selective dose of 8-SPT (10SPT), as well as by an A_3 selective dose of MRS-1191 (MRS), and abolished by combined pretreatment with the A_1 and A_3 antagonists or a non-selective dose of 8-SPT (50SPT). *P < 0.05 vs Sham; †P < 0.05 vs CAO15; ‡P < 0.05 vs 10SPT + CAO15 and vs MRS + CAO15.

Protocol III: Involvement of adenosine receptor stimulation in interorgan preconditioning.

Three cycles of 3-min mesenteric artery occlusion and 5 min of reperfusion (3- MAO3) failed to protect the myocardium (Figure 3), while a single 15-min MAO preceding a 60-min CAO by 10 min of reperfusion (MAO15) provided similar protection as CAO15. Pretreatment with the non-selective dose of 8-SPT (50SPT) abolished the cardioprotection by MAO15 (Figure 3). The A_1-selective dose of 8-SPT (10SPT) and the A_3-selective dose of MRS 1191 each attenuated the cardioprotection by MAO15 by approximately 65%, while combined pretreatment (10SPT + MRS) with the A_1- and A_3-selective doses of these antagonists abolished the cardioprotection.

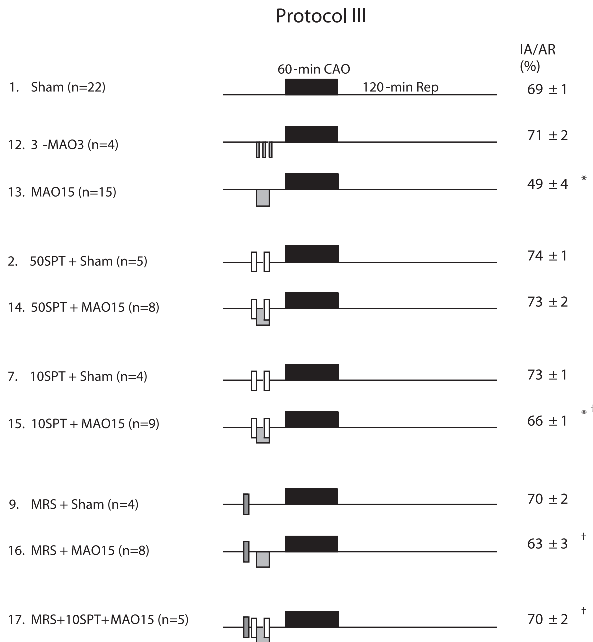

Figure 3. Cardioprotection by interorgan preconditioning (IOPC), and involvement of adenosine and A_1 and A_3-receptor subtypes in IOPC by MAO15. Both A_1 and A_3-adenosine receptor subtypes were required for IOPC by MAO15. Also note that whereas 3-CAO3 was more cardioprotective than CAO15 in classical preconditioning (see Figure 1), 3-MAO3 was ineffective in eliciting IOPC. *$P < 0.05$ vs Sham; †$P < 0.05$ vs MAO15.

Protocol IV: Effect of small intestinal adenosine receptor stimulation on myocardial infarct size by 60-min CAO.

Figure 4 shows that a 15 min adenosine infusion (10µg/min) into the mesenteric artery (ADO IMA), followed by 10 min washout, limited infarct size produced by a 60-min CAO (IS = 47 ± 4%) to the same extent as MAO15 (IS = 49 ± 4%). Pretreatment with hexamethonium abolished the cardioprotection by ADO IMA (Hex + ADO IMA), implicating the involvement of a neurogenic pathway in the cardioprotection by ADO IMA. This is also confirmed by the lack of effect of infusion of the same dose of adenosine into the portal vein (ADO IPV), excluding that (i) recirculation

of adenosine during ADO IMA stimulated cardiac adenosine receptors directly, and (ii) spillover of adenosine during ADO IMA stimulated adenosine receptors in the liver and contributed to the cardioprotection by ADO IMA.

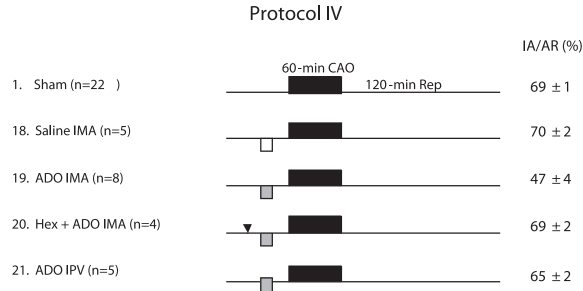

Figure 4. Involvement of small intestinal adenosine receptor stimulation in IOPC. Intramesenteric artery infusion of adenosine (ADO IMA) limited infarct size to the same extent as MAO15 (see Figure 3). The protection by ADO IMA was under neurogenic control as pretreatment with hexamethonium (Hex + ADO IMA) abolished the protection and intraportal adenosine infusion (ADO IPV) was ineffective. *P < 0.05 vs Sham. †P < 0.05 Hex + ADO IMA vs ADO IMA.

Protocol V: Involvement of myocardial adenosine receptor stimulation in IOPC.

Figure 5 shows that whereas pretreatment with hexamethonium abolished IOPC by MAO15 (Hex + MAO15, IS = 74 ± 2%), hexamethonium did not abrogate IOPC when administered at 5 min of mesenteric artery reperfusion (MAO15 + Hex, IS = S4 ± 2%). In Contrast, both pretreatment (50SPT + MAO15, IS = 73 ± 2%) and treatment at 5 min of mesenteric reperfusion with 8-SPT (MAO15 + 50SPT, IS = 67 ± 3%) abolished IOPC.

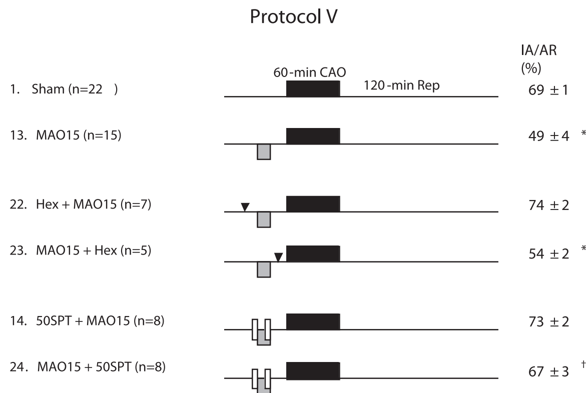

Figure 5. Involvement of myocardial adenosine receptor stimulation in IOPC by MAO15. Notice that, whereas treatment with hexamethonium 5 min after MAO15 (MAO15 + Hex) had no effect on IOPC, treatment with 8-SPT 5 min after MAO15 (MAO15 + 50SPT) abolished IOPC. *P < 0.05 vs Sham; †P < 0.05 vs MAO15.

DISCUSSION

Involvement of adenosine in IPC in the rat heart

The role of adenosine in ischemic preconditioning has been confirmed in several species such as rabbits [22], dogs [23] and swine [24, 25]. However, several groups of investigators have failed to show a contribution of adenosine to infarct size limitation by IPC in the rat heart, although pre-treatment with exogenous adenosine is cardioprotective in this species [4]. We hypothesized that the failure to establish the role of adenosine in the aforementioned studies in rats [4] could be due to the fact that the duration of the IPC stimulus may have been too short [6]. In the present study we confirmed that protection afforded by 3 cycles of 3-min CAO and 5 min of reperfusion was not amenable to adenosine receptor blockade with 8-SPT [2]. In contrast, when a preconditioning stimulus of 15min duration was used, the protection was already blunted by a dose of 8-SPT that was half (2 X 5 mg/kg) and abolished by a dose that was 2.5 times (2 x 25mg/kg) that was used by Li and Kloner [2]. These observations imply that adenosine receptor activation (both A_1- and A_3-subtypes) is involved in IPC, provided that the duration of the preconditioning stimulus is sufficiently long.

In interstitial fluid collected between 1 and 6 min of a 6-min of global normothermic ischemia in isolated hearts, myocardial interstitial adenosine concentrations increased from 0.25 to 6.8μM in rats but only from 0.33 to 2.0μM in rabbits [26]. The K_i value of adenosine for the A_1-receptor is 10-100nM in rats and 28nM in rabbits [4, 27]. In view of the three fold higher interstitial adenosine levels during ischemia in the rat compared to the rabbit heart, and the similar affinity for A_1-receptors in rat and rabbit hearts, it is difficult to explain why adenosine would not contribute to IPC in the rat heart [4]. Since Headrick [26] used a 6-min CAO, the different results obtained in rats and rabbits can only be explained by a more rapid increase in adenosine in the rabbits, whereas in rats interstitial adenosine levels do not increase sufficiently during the initial 3 min of occlusion to produce adequate adenosine receptor stimulation.

Since endogenous adenosine has a 10-100 fold higher affinity for the A_1-receptors over A_3-receptors, it is possible that in other species the A_3-receptor may not contribute to the same extent. Interestingly, the protection by hypoxic preconditioning against hypoxia-induced damage in isolated rabbit cardiomyocytes could only be partially blocked by the adenosine A_1-selective antagonist DPCDX, and required a combination of DPCPX with either the adenosine A_1/A_3 antagonist BWA1433 or with 8-SPT, for complete blockade, suggesting that also in the rabbit heart both adenosine A_1- and A_3-receptors might contribute to ischemic preconditioning in vivo [28].

IOPC stimulus

In our original study [7], MAO15 was as effective as a CAO15 in limiting myocardial infarct size produced by a subsequent 60-min CAO. Because 3-CAO3 afforded greater protection than CAO15 [12], we investigated whether 3-MAO3 was also more cardioprotective than MAO15. Our data show that these multiple brief MAO's were unable to precondition the myocardium.

Involvement of adenosine receptor stimulation in IOPC

The cardioprotection by small intestinal ischemia was completely prevented by pretreating rats with 50 mg/kg 8-SPT, implying that adenosine is at least one of the mediators leading to cardioprotection in this model of IOPC. The present study also demonstrates that, at least with the currently used stimulus, both the A_1- and A_3-receptor subtypes contribute. Adenosine may also play a role in IOPC by renal ischemia [16-18], but in none of these studies the location and the subtype of the involved adenosine receptors was investigated.

Myocardial adenosine receptors

Because the adenosine receptor antagonists were administered intravenously prior to MAO15, the data in Figure 3 do not reveal the site of action of adenosine. In order to investigate whether the myocardial adenosine receptors were involved, we administered 8-SPT after 5 min of mesenteric artery reperfusion because at this time point hexamethonium did not influence cardioprotection by IOPC, indicating that IOPC no longer required activation of the neurogenic pathway. 8-SPT administered at 5 min of mesenteric artery reperfusion abolished all cardioprotection by IOPC, implying that the myocardial adenosine receptors must be involved in the mechanism underlying this phenomenon. The myocardial adenosine receptors involved are of both the A_1- and A_3-subtypes [21], which is in agreement with our findings in classical IPC in protocol II.

Small intestinal adenosine receptors

ADO IMA limited myocardial infarct size produced by 60-min CAO to the same extent as MAO15. Moreover, because infusion of the same dose of adenosine in the portal vein did not protect the myocardium, the adenosine must have acted in the small intestine. The protection by locally administered adenosine was abolished after pretreatment with hexamethonium, implying that the neurogenic pathway was also involved in this model of pharmacological IOPC. The intramesenteric artery infusion rate was chosen such that only the adenosine receptors in the small intestine were activated. However, we do not know how the adenosine concentrations in the small intestine achieved during intramesenteric artery infusion compare to the concentrations that are achieved during MAO15. Therefore, it cannot be excluded that in the portal vein higher concentrations were reached upon reperfusion of the ischemic small intestine than during the direct infusion of adenosine into the portal vein. If true, these higher concentrations might have been sufficient to stimulate afferent nerves in the liver and thereby have contributed to the cardioprotection by small intestine ischemia. The observation that the protection by ADO IMA was very similar to that by MAO15 suggests that the concentrations during ADO IMA and MAO15 were also similar.

To definitively demonstrate the involvement of adenosine receptor stimulation in the small intestine in IOPC requires blockade of intestinal receptors, while leaving the myocardial receptors unaffected. Because of the half-life of the antagonists used in the present study, recirculation will lead to adenosine receptor blockade in the heart. Nevertheless, the present observations are consistent with the hypothesis that in IOPC by small intestinal ischemia, locally released adenos-

ine triggers afferent nerves which in turn lead to stimulation of myocardial adenosine receptors. Adenosine is currently under investigation for its purported ischemia reperfusion damage limiting effects [29, 30]. The results of the present study suggest that the cardioprotective effects of intravenous adenosine may not only be due to direct effects in the heart but could at least in part result from adenosine receptor activation in remote tissues which via neurogenic pathways increase interstitial adenosine levels in the heart.

ACKNOWLEDGEMENTS

This study was supported by Netherlands Heart Foundation Grants NHS 95.103, NHS 99.143, and an "Established Investigator" Stipend 2000D038 from the Netherlands Heart Foundation (DJ Duncker).

REFERENCE

1. LIU, Y. AND J.M. DOWNEY, ISCHEMIC PRECONDITIONING PROTECTS AGAINST INFARCTION IN RAT HEART. AM J PHYSIOL, 1992. 263(4 PT 2): P. H1107-12.
2. LI, Y. AND R.A. KLONER, THE CARDIOPROTECTIVE EFFECTS OF ISCHEMIC 'PRECONDITIONING' ARE NOT MEDIATED BY ADENOSINE RECEPTORS IN RAT HEARTS. CIRCULATION, 1993. 87(5): P. 1642-8.
3. BUGGE, E. AND K. YTREHUS, ISCHAEMIC PRECONDITIONING IS PROTEIN KINASE C DEPENDENT BUT NOT THROUGH STIMULATION OF ALPHA ADRENERGIC OR ADENOSINE RECEPTORS IN THE ISOLATED RAT HEART. CARDIOVASC RES, 1995. 29(3): P. 401-6.
4. GANOTE, C.E. AND S.C. ARMSTRONG, ADENOSINE AND PRECONDITIONING IN THE RAT HEART. CARDIOVASC RES, 2000. 45(1): P. 134-40.
5. WHITTAKER, P., R.A. KLONER, AND K. PRZYKLENK, INTRAMYOCARDIAL INJECTIONS AND PROTECTION AGAINST MYOCARDIAL ISCHEMIA. AN ATTEMPT TO EXAMINE THE CARDIOPROTECTIVE ACTIONS OF ADENOSINE. CIRCULATION, 1996. 93(11): P. 2043-57.
6. SCHULZ, R., ET AL., ISCHEMIC PRECONDITIONING IN PIGS: A GRADED PHENOMENON: ITS RELATION TO ADENOSINE AND BRADYKININ. CIRCULATION, 1998. 98(10): P. 1022-9.
7. GHO, B.C., ET AL., MYOCARDIAL PROTECTION BY BRIEF ISCHEMIA IN NONCARDIAC TISSUE. CIRCULATION, 1996. 94(9): P. 2193-200.
8. VAN DEN DOEL, M.A., ET AL., HYPOTHERMIA EXTENDS THE CARDIOPROTECTION BY ISCHAEMIC PRECONDITIONING TO CORONARY ARTERY OCCLUSIONS OF LONGER DURATION. CARDIOVASC RES, 1998. 37(1): P. 76-81.
9. RICE, P.J., S.C. ARMSTRONG, AND C.E. GANOTE, CONCENTRATION-RESPONSE RELATIONSHIPS FOR ADENOSINE AGONISTS DURING PRECONDITIONING OF RABBIT CARDIOMYOCYTES. J MOL CELL CARDIOL, 1996. 28(6): P. 1355-65.
10. LIU, G.S., ET AL., EVIDENCE THAT THE ADENOSINE A3 RECEPTOR MAY MEDIATE THE PROTECTION AFFORDED BY PRECONDITIONING IN THE ISOLATED RABBIT HEART. CARDIOVASC RES, 1994. 28(7): P. 1057-61.
11. AUCHAMPACH, J.A., ET AL., SELECTIVE ACTIVATION OF A3 ADENOSINE RECEPTORS WITH N6-(3-IODOBENZYL) ADENOSINE-5'-N-METHYLURONAMIDE PROTECTS AGAINST MYOCARDIAL STUNNING AND INFARCTION WITHOUT HEMODYNAMIC CHANGES IN CONSCIOUS RABBITS. CIRC RES, 1997. 80(6): P. 800-9.
12. LIEM, D.A., ET AL., ROLE OF ADENOSINE IN ISCHEMIC PRECONDITIONING IN RATS DEPENDS CRITICALLY ON THE DURATION OF THE STIMULUS AND INVOLVES BOTH A(1) AND A(3) RECEPTORS. CARDIOVASC RES, 2001. 51(4): P. 701-8.
13. PRZYKLENK, K., ET AL., REGIONAL ISCHEMIC 'PRECONDITIONING' PROTECTS REMOTE VIRGIN MYOCARDIUM FROM SUBSEQUENT SUSTAINED CORONARY OCCLUSION. CIRCULATION, 1993. 87(3): P. 893-9.
14. TANG, Z.L., ET AL., INVOLVEMENT OF CAPSAICIN-SENSITIVE SENSORY NERVES IN EARLY AND DELAYED CARDIOPROTECTION INDUCED BY A BRIEF ISCHAEMIA OF THE SMALL INTESTINE. NAUNYN SCHMIEDEBERGS ARCH PHARMACOL, 1999. 359(3): P. 243-7.
15. XIAO, L., ET AL., DELAYED CARDIOPROTECTION BY INTESTINAL PRECONDITIONING IS MEDIATED BY CALCITONIN GENE-RELATED PEPTIDE. EUR J PHARMACOL, 2001. 427(2): P. 131-5.
16. DING, Y.F., M.M. ZHANG, AND R.R. HE, ROLE OF RENAL NERVE IN CARDIOPROTECTION PROVIDED BY RENAL ISCHEMIC PRECONDITIONING IN ANESTHETIZED RABBITS. SHENG LI XUE BAO, 2001. 53(1): P. 7-12.
17. PELL, T.J., ET AL., RENAL ISCHEMIA PRECONDITIONS MYOCARDIUM: ROLE OF ADENOSINE RECEPTORS AND ATP-SENSITIVE POTASSIUM CHANNELS. AM J PHYSIOL, 1998. 275(5 PT 2): P. H1542-7.
18. TAKAOKA, A., ET AL., RENAL ISCHEMIA/REPERFUSION REMOTELY IMPROVES MYOCARDIAL ENERGY METABOLISM DURING MYOCARDIAL ISCHEMIA VIA ADENOSINE RECEPTORS IN RABBITS: EFFECTS OF "REMOTE PRECONDITIONING". J AM COLL CARDIOL, 1999. 33(2): P. 556-64.
19. BIRNBAUM, Y., S.L. HALE, AND R.A. KLONER, ISCHEMIC PRECONDITIONING AT A DISTANCE: REDUCTION OF MYOCARDIAL INFARCT SIZE BY PARTIAL REDUCTION OF BLOOD SUPPLY COMBINED WITH RAPID STIMULATION OF THE GASTROCNEMIUS MUSCLE IN THE RABBIT. CIRCULATION, 1997. 96(5): P. 1641-6.
20. DE ZEEUW, S., ET AL., CARDIOPROTECTION IN PIGS BY EXOGENOUS NOREPINEPHRINE BUT NOT BY CEREBRAL ISCHEMIA-INDUCED RELEASE OF ENDOGENOUS NOREPINEPHRINE. STROKE, 2001. 32(3): P. 767-74.
21. LIEM, D.A., ET AL., SITES OF ACTION OF ADENOSINE IN INTERORGAN PRECONDITIONING OF THE HEART. AM J PHYSIOL HEART CIRC PHYSIOL, 2002. 283(1): P. H29-37.
22. LIU, G.S., ET AL., PROTECTION AGAINST INFARCTION AFFORDED BY PRECONDITIONING IS MEDIATED BY A1 ADENOSINE RECEPTORS IN RABBIT HEART. CIRCULATION, 1991. 84(1): P. 350-6.

23. AUCHAMPACH, J.A. AND G.J. GROSS, ADENOSINE A1 RECEPTORS, KATP CHANNELS, AND ISCHEMIC PRECONDITIONING IN DOGS. AM J PHYSIOL, 1993. 264(5 PT 2): P. H1327-36.
24. VAN WINKLE, D.M., ET AL., CARDIOPROTECTION PROVIDED BY ADENOSINE RECEPTOR ACTIVATION IS ABOLISHED BY BLOCKADE OF THE KATP CHANNEL. AM J PHYSIOL, 1994. 266(2 PT 2): P. H829-39.
25. SCHULZ, R., ET AL., INVOLVEMENT OF ENDOGENOUS ADENOSINE IN ISCHAEMIC PRECONDITIONING IN SWINE. PFLUGERS ARCH, 1995. 430(2): P. 273-82.
26. HEADRICK, J.P., ISCHEMIC PRECONDITIONING: BIOENERGETIC AND METABOLIC CHANGES AND THE ROLE OF ENDOGENOUS ADENOSINE. J MOL CELL CARDIOL, 1996. 28(6): P. 1227-40.
27. JACOBSON, K.A., ADENOSINE A3 RECEPTORS: NOVEL LIGANDS AND PARADOXICAL EFFECTS. TRENDS PHARMACOL SCI, 1998. 19(5): P. 184-91.
28. WANG, J., ET AL., DUAL ACTIVATION OF ADENOSINE A1 AND A3 RECEPTORS MEDIATES PRECONDITIONING OF ISOLATED CARDIAC MYOCYTES. EUR J PHARMACOL, 1997. 320(2-3): P. 241-8.
29. MAHAFFEY, K.W., ET AL., ADENOSINE AS AN ADJUNCT TO THROMBOLYTIC THERAPY FOR ACUTE MYOCARDIAL INFARCTION: RESULTS OF A MULTICENTER, RANDOMIZED, PLACEBO-CONTROLLED TRIAL: THE ACUTE MYOCARDIAL INFARCTION STUDY OF ADENOSINE (AMISTAD) TRIAL. J AM COLL CARDIOL, 1999. 34(6): P. 1711-20.
30. KLONER, R.A. AND R.B. JENNINGS, CONSEQUENCES OF BRIEF ISCHEMIA: STUNNING, PRECONDITIONING, AND THEIR CLINICAL IMPLICATIONS: PART 2. CIRCULATION, 2001. 104(25): P. 3158-67.

Chapter 6

Amelioration of renal ischemia reperfusion injury by synthetic oligopeptides related to human chorionic gonadotropin

NA Khan*, D Susa, JW van den Berg, M Huisman, MH Ameling*, S van den Engel, HP Roest, JNM IJzermans, WA Dik*, R Benner*, RWF de Bruin

Departments of Surgery and Immunology*, Erasmus Medical Center, Rotterdam, The Netherlands

These authors (D Susa, NA Khan) contributed equally to this work

Nephrology, Dialysis and Transplantation 2009; 24 (9): 2701-8

ABSTRACT

We have previously reported that small synthetic oligopeptides related to human ß-chorionic gonadotropin (ß-hCG) can reduce inflammation. Here we investigated whether such oligopeptides can reduce renal ischemia reperfusion injury in the mouse.

Ten different oligopeptides were administered 1 minute before induction of renal ischemia and 1 minute before reperfusion.

Survival at 72 hours post reperfusion was significantly higher in mice treated with oligopeptides MTRV, LQG, VLPALPQ or AQGV as compared to placebo treated mice. Some oligopeptides were more effective than others. AQGV completely prevented mortality and best preserved kidney function. Next, AQGV was tested in a dose-escalating study in a range of 0.3 to 30 mg/kg. A survival gain was observed with all doses. Improvement of kidney function was observed from 1 mg/kg. Highest survival and best preserved kidney function was observed at 3 and 10 mg/kg.

Upon treatment with AQGV, a significantly lower influx of neutrophils was found, apoptosis was decreased, whereas tubular epithelial cell proliferation was significantly increased at 24 hrs post-reperfusion. Serum levels of TNF-α, INF-γ, IL-6 and IL-10 were significantly decreased at 24 hours post reperfusion. E-selectin mRNA levels in kidneys were significantly decreased at 6 hours post-reperfusion. AQGV did not reduce mortality when treatment was started after reperfusion.

This study shows that small oligopeptides related to the primary structure of β-hCG, especially AQGV, are promising potential drugs for preventing the development of renal ischemia reperfusion injury.

INTRODUCTION

Inflammation plays a major role in the pathophysiology of renal ischemic reperfusion (I/R) injury [1]. The initial ischemic injury results in up-regulation of adhesion molecules on activated endothelium and release of cytokines, reactive oxygen species (ROS) and eicosanoids. Leukocytes, recruited by chemokines and pro-inflammatory cytokines, potentiate injury by generating more ROS and eicosanoids, thereby further enhancing inflammation.

Human chorionic gonadotropin (hCG) is a hormone produced during pregnancy by placental trophoblasts [2], but is also produced by the pituitary gland and leukocytes in non-pregnant females and males [3,4]. It consists of an α- and β-chain (Figure1). In human pregnancy urine and in commercial hCG preparations hCG occurs in a variety of forms, including breakdown oligopeptide products. During pregnancy, the urine contains increasing proportions of nicked hCG and hCG β-core fragments [5]. Nicked hCG has peptide bond cleavages in loop 2 of the β-chain between residues 44 and 52, whereas hCG β-core completely lacks the β-chain loop 2, which consists of amino acid residues 41-54.

We have previously shown that the 400 – 2000 Dalton peptide fraction of pregnancy urine, but not of normal female or male urine, is able to inhibit the development of diabetes in NOD mice, whereas fractions greater than 2000 Dalton, including hCG, did not have this activity [6]. We then reported that the synthetic hexapeptide VLPALP, which is part of the primary structure of the hCG β-chain loop 2, reduced mortality in a murine model of lipopolysacharide (LPS) induced systemic inflammatory response syndrome [7,8].

Based on these findings, and known preferential cleavage sites of the hCG ß-chain loop 2 [5,9-12], we selected six different synthetic oligopeptides (MTR, MTRV, LQG, LQGV, VLPALP and VLPALPQ), which are part of the primary structure of loop 2 of the hCG ß-chain, as well as four alanine variants of LQG and LQGV (AQG, LAG, AQGV and LAQV) and tested these in a murine model for their capacity to reduce renal I/R injury.

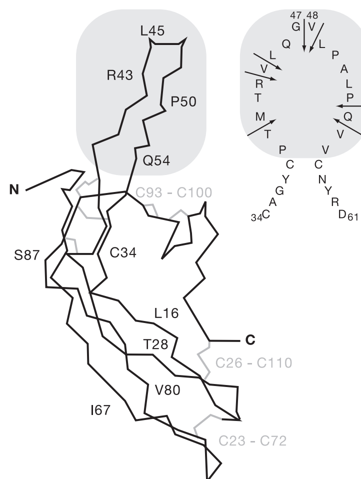

Figure 1. Structure of β-hCG with loop 2 and the amino acid sequence of loop 2 indicated. Adapted from Lapthorn et al. [35]. Arrows indicate the preferential cleavage sites in loop 2.

MATERIALS AND METHODS

Experimental design

The experimental protocol was approved by the Animal Experiments Committee under the Dutch Experiments on Animals Act and adhered to the rules laid down in this national law that serves the implementation of "Guidelines on the protection of experimental animals" by the Council of Europe (1986), Directive 86/609/EC.

Ten different hCG-related oligopeptides (MTR, MTRV, LQG, LQGV, VLPALP, VLPALPQ, AQG, AQGV, LAG and LAGV) were evaluated for their capacity to reduce ischemia reperfusion induced renal injury as compared to mice treated with phosphate-buffered saline (PBS). Five mg/kg body weight of oligopeptide or PBS in a volume of 0.1 mL was administered intravenously (i.v.) 1 minute before clamping the kidney, and 1 minute before releasing the clamp.

Subsequently a dose-escalating study was performed with AQGV. The AQGV was given in doses of 0.3, 1, 3, 10, and 30 mg/kg in a volume of 0.1 mL and was administered i.v. 1 minute before clamping, and 1 minute before releasing the clamp. Possible toxic side effects were studied by careful observation of control and peptide treated mice for signs of discomfort.

Contra-lateral kidney samples were obtained for further analysis. At 24, and 72 hr post-reperfusion, mice were sacrificed and clamped kidneys were harvested and snap frozen for further analysis. Serum urea levels were measured to determine kidney function. Infiltrating cells were analysed using immunohistochemistry. In all groups survival was assessed and analyzed by Kaplan-Meier analysis.

In an additional experiment AQGV was given in a dose of 5 mg/kg BW in a volume of 0.1 mL and administered i.v. 1 minute before clamping, and 1 minute before releasing the clamp. At 6 and 24 hr post-reperfusion, mice were sacrificed and blood was obtained for cytokine measurements in serum. From the 6hr post-reperfusion group the clamped kidney was harvested for determination of mRNA expression levels.

Furthermore, survival experiments were performed in which mice received PBS or AQGV (5mg/kg BW) at 12 and 24 hours, or at 6 and 12 hours post-reperfusion.

Mice

Male C57BL/6JOlaHsd mice of 12-16 weeks of age were obtained from Harlan (Horst, The Netherlands). Mice were kept under standard laboratory conditions (temperature 20-24ºC, relative humidity 50-60%, 12 h light/12 h dark) and were allowed free access to food (Hope Farms, Woerden, The Netherlands) and water.

Ischemia model

Mice were anaesthetized by isoflurane inhalation. Anaesthesia was maintained using a mixture of N_2O/O_2/isoflurane. Blood was collected by retro-orbital puncture. Body temperature was maintained by placing the mice on heating pads. Following a midline abdominal incision, the left renal pedicle was localized and clamped for 25 minutes using an atraumatic micro-vascular clamp. After inspection for signs of ischemia, the wound was covered with PBS soaked cotton and the animal was covered with a tin foil insulation sheet. After release of the clamp, restoration of blood-flow

was inspected visually and a contra-lateral nephrectomy was performed. The abdominal wound was closed in two layers, and mice were given 0.5 ml PBS subcutaneously.

Oligopeptides

Selection was based on either the known preferential cleavage sites or known in vivo nick sites of the sequence MTRVLQGVLPALPQ (aa_{41-54}) of loop 2 of the β-subunit of hCG [14, 16-19]. Selected oligopeptides were MTR (aa_{41-43}), MTRV (aa_{41-44}), LQG (aa_{45-47}), AQG and LAG (alanine replaced oligopeptides of LQG), LQGV (aa_{45-48}), AQGV and LAGV (alanine replaced oligopeptide of LQGV), VLPALP (aa_{48-53}), VLPALPQ (aa_{48-54}). Oligopeptides were synthesized (Ansynth BV, Roosendaal, The Netherlands) using the fluorenylmethoxycarbonyl (Fmoc)/tert-butyl-based methodology with a 2-chlorotritylchloride resin as the solid support. Oligopeptides were dissolved in PBS at a concentration of 1 mg/ml and stored at -20°C in small aliquods.

Functional measurements

Serum urea and creatinine values were measured using a kinetic urease method where the decrease in NADH absorbance is measured photometrically, using an ELAN multianalyser (Eppendorf-Merck, Germany).

Immunohistochemistry

Primary antibodies used were rat-anti-mouse CD4, CD8, CD45, neutrophils, macrophages, CD54 (Serotec, Oxford, UK). Antibodies were diluted in PBS/5% BSA solution. Primary antibody was applied on for 30 min at RT and slides were subsequently incubated with a mixture of goat-anti-rat IgM+IgG (H+L) alkaline-phosphate conjugated antibody (Southern Biotech, Birmingham, USA) for 30 min at RT. Enzyme detection was performed using Naphthol AS-MX, New Fuchsine, sodium-nitrite and levamisol mixture in Tris-HCl pH8 as a substrate for 30 min at RT in the dark.
Formalin-fixed-paraffin sections (3 μm) were used for Ki-67 staining. Slides were deparaffinized and rehydrated and boiled for antigen retrieval in a 0.01M sodium citrate solution for 30 min in a microwave-oven. Endogenous peroxidase was blocked with a 0.03% H_2O_2 solution. The sections were incubated overnight at 4°C with rat-anti-mouse Ki-67 primary antibody (Dako Cytomation, Glostrup, Denmark) and subsequently incubated for 30 min at RT with rabbit-anti-rat IgG conjugated with HRP secondary antibody (Dako Cytomation, Glostrup, Denmark). Enzyme detection was performed using DAB as a substrate. Slides were rinsed in tap water, counterstained with haematoxylin and rinsed with tap water again. As a negative control the primary antibody was omitted. Positive cells were counted in 10 high power fields (400X) using a semi quantitative scoring system as follows: 0: no positive cells, 1: 1-10 cells, 2: 11-30 cells, 3: 30-60 cells, 4 > 60 cells.

Measurement of apoptotic cells

Formalin-fixed-paraffin sections were stained for apoptotic cells by TUNEL staining using the ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Millipore, USA) according to the manufacturers instructions. Positive cells were counted in 10 fields at a magnification of 400x.

Cytokine measurements

TNF-α, IFN-γ, IL-6, IL-10, IL-12, and MCP-1 were measured using a commercially available cytometic bead array (CBA) (BD Biosciences, San Jose, CA, USA) and a BD FACSArrayTM Bioanalyzer (BD Biosciences). Analysis of the data was performed using FCAP ArrayTM software (BD Biosciences). Assay sensitivity was 2.5 pg/ml.

Real-time quantitative (RQ)-PCR analysis

Sections of kidney were homogenized and RNA was isolated using the Qiagen RNeasy kit (Qiagen, Hilden, Germany). In total 1μg of RNA was reverse transcribed and RQ-PCR using an AppliedBiosystems 7700 PCR machine (Foster City, CA, USA) was performed as described previously [13]. In all 6h samples the mRNA transcript levels of TNF-α, IFN-γ, IL-6, IL-10, IL-12, MCP-1, and the adhesion molecules E-selectin and ICAM-1 were determined. Transcript levels of these genes were quantified by normalization against ABL.

Statistical analysis

Survival data were compared by log-rank analysis. Other data were analysed using ANOVA, followed by a Mann-Whitney – U test. Calculations were performed using SPSS v11.0 for Windows. A p value≤0.05 was considered statistically significant. Data are presented as mean values ± standard error of the mean.

RESULTS

Effect of hCG-related oligopeptide treatment on survival

25 minutes of warm renal ischemia and contra-lateral nephrectomy resulted in a survival of 50% in the control group at 3 days post-reperfusion (Table 1). The groups treated with oligopeptides MTR, LQGV, VLPALP, AQG, LAG and LAGV (5 mg/kg), had survival rates not significantly different from controls. Treatment with LQG led to a significant better survival (90%), while treatment with oligopeptides MTRV, VLPALPQ or AQGV totally prevented mortality.

	Survival		
Treatment	24h	72h	P
PBS	90%	50%	
MTR	100%	60%	ns
MTRV	100%	100%	< 0.05
LQG	100%	90%	< 0.05
LQGV	100%	80%	ns
VLPALP	100%	70%	ns
VLPALPQ	100%	100%	< 0.01
AQG	100%	70%	ns
AQGV	100%	100%	< 0.01
LAG	100%	70%	ns
LAGV	90%	90%	ns

Table 1. Effect of various hCG-related oligopeptides (5 mg/kg) on the survival of mice subjected to ischemia reperfusion damage

Effect of hCG-related oligopeptide treatment on kidney function

Treatment of mice with oligopeptide MTRV, AQGV or LAGV provided significant ($p<0.05$) functional protection against renal I/R injury at both 24 and 72 hr, as measured by serum urea levels (Figure 2). Although treatment with LQG resulted in significantly decreased serum urea at 24 hours post-reperfusion ($p<0.05$), at 72 hours no significant beneficial effect was found. While treatment with VLPALPQ did not cause a significant decrease in serum urea at 24 hours, at 72 hours it was significantly decreased as compared to the control group ($p<0.05$). Treatment with AQGV provided the most powerful protection against renal ischemia reperfusion injury at both 24 hours ($p<0.01$) and 72 hours ($p<0.01$).

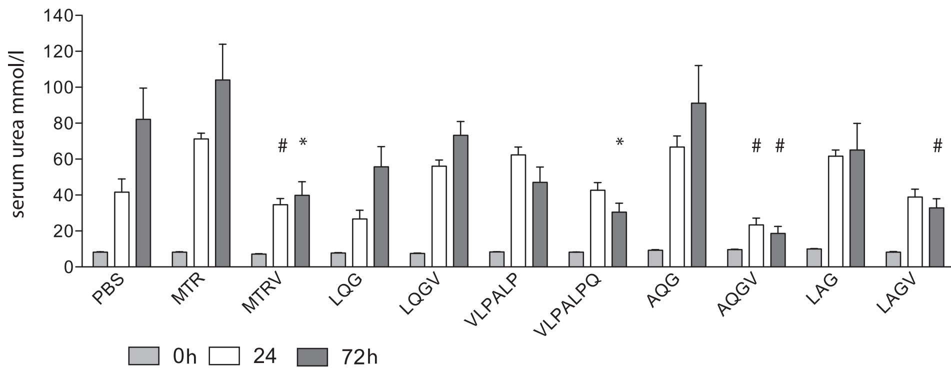

Figure 2. Renal function as reflected by serum urea levels. Pre-operative values and 24 and 72 hours post reperfusion values in the different oligopeptide treated groups were compared to PBS-treated controls. Treatment with MTRV and AQGV significantly reduced renal function loss both at 24 hours and 72 hours after ischemia reperfusion injury. Treatment with LQG reduced serum urea levels at 24 hours. Treatment with VLPALPQ and LAGV showed significantly reduced serum urea levels at 72 hours. *$p<0.05$ and #$p<0.01$ (n = 10 animals / group).

Effect of different doses of AQGV (0.3 – 30 mg/kg) on survival

Because AQGV showed the most powerful protection against warm renal I/R injury, we determined the optimal dose of this oligopeptide in a dose escalating study. Therefore, AQGV was administered in doses ranging from 0.3 to 30 mg/kg, and compared to mice treated with PBS. A survival rate of 60% was seen in the control group (Table 2). Although treatment with 0.3, 1 and 30 appeared to result in a survival benefit, no significant difference could be measured (80%, 90%, and 80%, respectively). The doses of 3 and 10 mg/kg totally prevented mortality ($p<0.05$).

	Survival		
Treatment (mg/kg)	24h	72h	P
PBS	100%	60%	
0.3	100%	80%	ns
1.0	100%	90%	ns
3.0	100%	100%	< 0.05
10	100%	100%	< 0.05
30	100%	80%	ns

Table 2. Effect of different doses of AQGV on the survival of mice subjected to ischemia reperfusion damage.

Effect of different doses of AQGV (0.3 – 30 mg/kg) on kidney function

Treatment of mice subjected to renal I/R damage with 1, 3, 10 and 30 mg/kg AQGV resulted in significant reduction of serum urea levels at 72 hours ($p<0.05$). A dose of 3 mg/kg resulted in best preservation of kidney function, with return to normal function already observed at 72 hrs ($p<0.01$). With 0.3 mg/kg no significant benefit was observed (Figure 3). Creatinine values confirmed these data but in our model did not show the same level of responsiveness to the injury as urea.

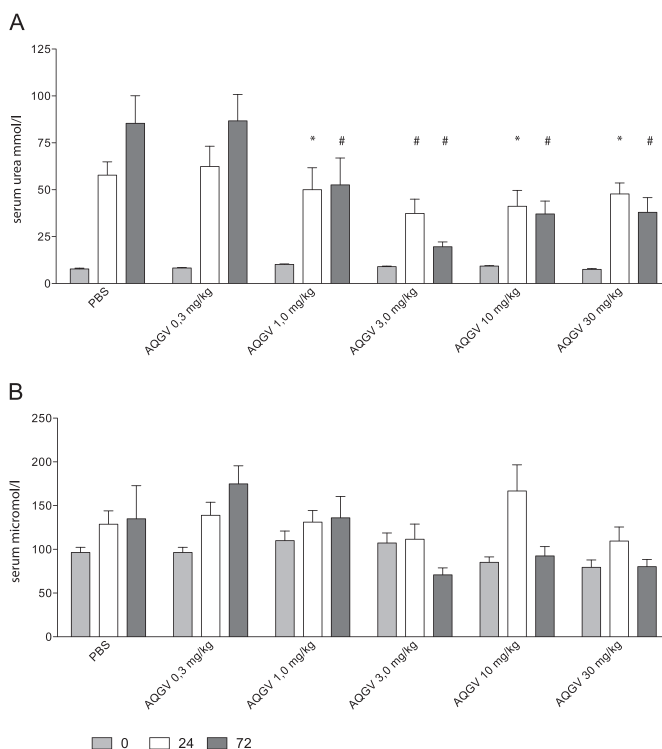

Figure 3. Renal function as reflected by serum urea (A) and creatinine (B). Values are shown pre-operative, and after 24 and 72 hours in groups treated with AQGV in a dose escalation study (0.3 – 30 mg/kg), and compared to a PBS-treated control group. Treatment with AQGV in a dose from 1 mg/kg up to 30 mg/kg significantly reduced renal function loss after renal ischemia reperfusion injury. The dose of 3 mg/kg was the most potent. *$p<0.05$ and #$p<0.01$ (n = 10 animals / group).

Effects of AQGV on cellular infiltration, apoptosis and proliferation.

To study the mechanism underlying the protective effect of AQGV, we investigated the cellular infiltrate and proliferation in the kidneys of mice treated with 5 mg/kg AQGV. At both 24 and 72 hr post reperfusion the neutrophil influx was significantly decreased in the AQGV-treated group ($p=0.03$ and $p=0.022$, respectively) (Figure 4A). Additional staining for CD4+, CD8+ cells and macrophages revealed no differences between the two groups (data not shown). TUNEL staining

identified apoptotic cells which were localized mainly in the tubular epithelium (Figure 4B, middle and lower panels). The number of TUNEL-positive cells was significantly lower in AQGV treated animals 24 hours after reperfusion (Figure 4B). Ki-67 staining showed a significantly higher proliferative activity of renal tubular epithelial cells in AQGV-treated mice at 24 hours (Figure 4C). At 72 hours this difference had disappeared

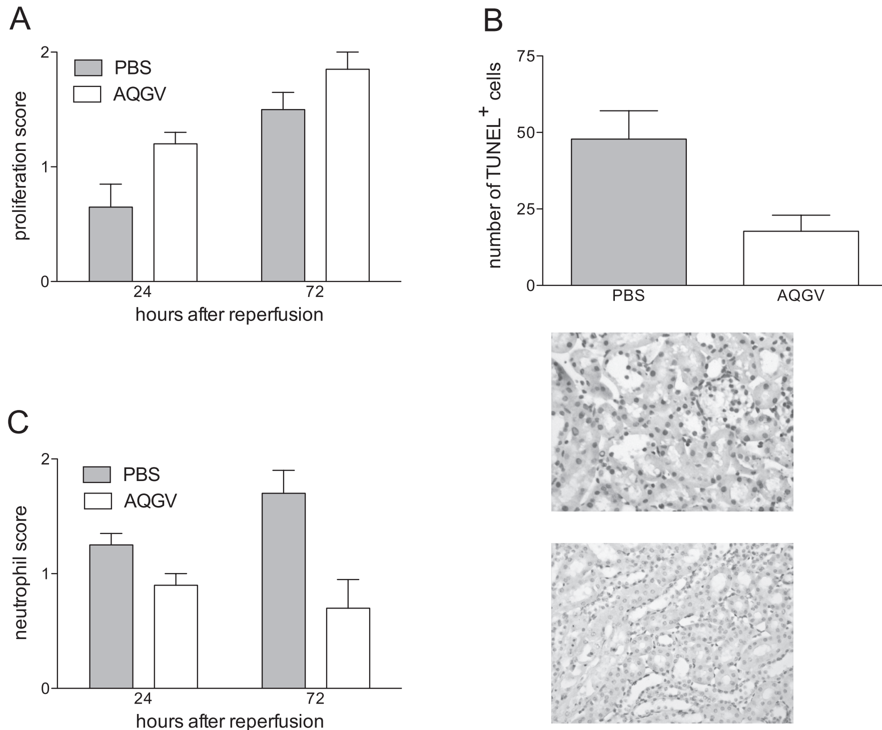

Figure 4. A. Renal neutrophil influx as assessed by immunohistochemical staining. AQGV treatment reduced neutrophil infiltration after 25 minutes of renal warm ischemia as assessed at 24 and 72 hours post-reperfusion. Data are expressed in a semi-quantitative way as described in the Material and Methods section. *p<0.05 (n = 10 animals / group). B. AQGV treatment significantly reduced the number of apoptotic cells in the kidney 24 hours after renal ischemia reperfusion injury. *p=0.01 vs PBS treated controls at 24 hours (n= 6 - 10 animals / group, upper panel). Middle and lower panels: representative photomicrographs of TUNEL stained control-, and AQGV treated kidneys respectively, 24 hours after reperfusion (200x). C. Proliferation as assessed by Ki-67 immunohistochemistry. AQGV treatment significantly enhanced cellular proliferation at 24 hours after renal ischemia reperfusion injury. Although a higher trend of proliferation was seen at 72 hours as well, no statistically significant difference was found. Data are expressed in a semi-quantitative way as described in the Material and Methods section. *p<0.05 (n = 10 animals / group).

Effects of AQGV on serum cytokine levels and renal mRNA transcript levels

Using the bead-array we determined serum cytokine levels at 6 and 24 hr post-reperfusion. MCP-1 was below the detection limit in all samples. No differences in serum TNF-α, IFN-γ, IL-6, IL-10, and IL-12 levels were observed at 6hrs post-reperfusion. At 24 hrs post-reperfusion the levels for all cytokines were decreased upon AQGV treatment, with IL-6, IL-10, IFN-γ ($p < 0.05$), and TNF-α ($p < 0.01$) being significantly lower (Figure 5A).

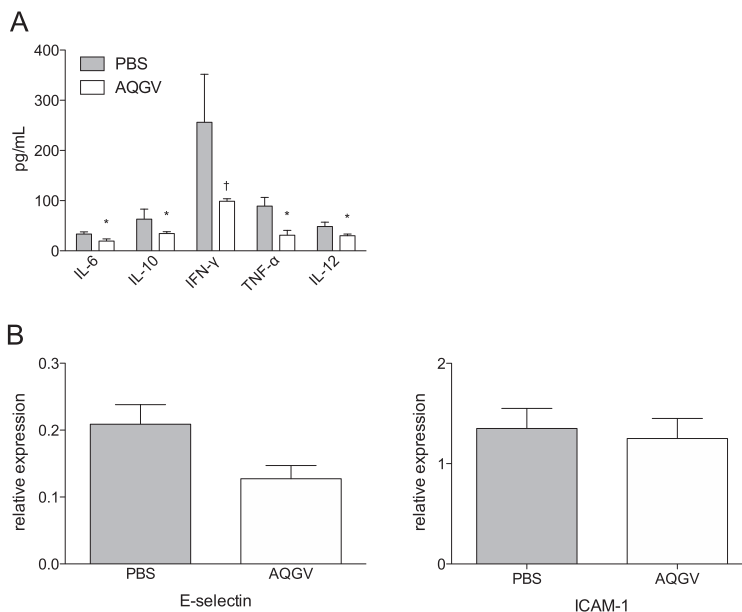

Figure 5. A. Treatment with AQGV reduces serum cytokine levels at 24 hours after renal ischemia reperfusion injury. *p<0.05, †p<0.01 (n = 5 per group). B. Treatment with AQGV reduces renal E-selectin (left panel) but not ICAM-1 (right panel) mRNA levels at 6 hours after renal ischemia reperfusion injury. Data are presented as mean value ± sem. *p<0.05 (n = 6 animals / group).Effect of post-reperfusion AQGV treatment on survival

AQGV treatment showed no effect on inflammatory cytokine mRNA levels 6 hrs post-reperfusion (data not shown). AQGV treatment did result in a significant ($p < 0.05$) down regulation of renal E-selectin, but not ICAM-1 mRNA expression at 6 hr post-reperfusion as compared to PBS treated mice (Figure 5B).

AQGV treatment given either at 12 and 24 hr post-reperfusion or at 6 and 12 hr post-reperfusion did not improve survival (~50%) as compared to the control group (data not shown).

DISCUSSION

We investigated whether treatment with synthetic oligopeptides, consisting of 3 to 7 amino acids, based on the primary structure of hCG, was able to reduce warm ischemia reperfusion injury of the kidney. We demonstrate for the first time that oligopeptides as small as three or four amino

acids can significantly reduce mortality seen after severe renal I/R injury and improves kidney function as measured by serum urea levels. Especially AQGV showed superior results in enhancing survival and preservation of kidney function after 25 minutes of renal ischemia. A dose of 3-10 mg/kg proved to be the most potent with regard to reducing mortality as well as preserving kidney function. Furthermore, up to 30 mg/kg, no toxicity was observed. Also in rats, dogs and a human phase I study no harmful side effects of single and repeated AQGV administration were found. Data of these studies will be published elsewhere (manuscript in preparation).

Both natural hCG and commercial hCG preparations have been investigated for their role on the immune system, because of their putative immunomodulating role during pregnancy in protecting the fetus from rejection [14].

Our previous work [6] shows that short-term treatment of female NOD mice, with a hCG preparation purified from first trimester pregnancy urine, starting prior to the onset of hyperglycemic symptoms, inhibits the development of type I diabetes. Interestingly, however, the anti-diabetic activity of the used hCG preparation did not reside in the heterodimeric hCG molecule, or its subunits, but in a 400-2000 Dalton fraction.

Subsequently, we showed in a model of LPS-induced systemic inflammatory response syndrome in mice that treatment with this low weight molecular fraction was capable of inhibiting the septic shock morbidity as well as mortality [7]. The same beneficial effect was obtained with the synthetic oligopeptide VLPALP, which sequence is part of loop 2 of the β-chain of hCG [7]. Recently, we showed that hCG-related oligopeptides reduce inflammation and liver injury in a rat model of hemorrhagic shock and resuscitation [15].

During pregnancy, hCG occurs in a variety of forms and breakdown products in serum and urine, including intact hCG, α- and β-subunits, nicked hCG, hCG β-core fragment, and smaller peptide fragments. Both nicked hCG and the β-core subunit consist of a β-chain with a defective loop 2. This loop, consisting of the amino acid residues 41-54, is absent in β-core subunit, and is cleaved in nicked hCG [9-12]. Since the immunomodulatory activity of hCG resided in the low molecular weight fraction, we hypothesised that in vivo liberated breakdown products, such as those originating from the proteolytic cleavage of peptide bonds between amino acid residues 41-54, may have significant biological activity [8]. Based on known preferential cleavage sites [5,9,10,12], we tested synthetic oligopeptides MTR, MTRV, LQG, LQGV, VLPALP, VLPALPQ and, based on alanine replacement mapping, the LQG and LQGV analogs AQG, LAG, AQGV and LAGV (Figure 1). Of these oligopeptides, MTRV, LQG, VLPALPQ and AQGV appeared able to reduce mortality and decline in kidney function induced by warm renal ischemia reperfusion injury, AQGV being the most effective (Table 1 and Figure 1).Cell migration plays an important role during the initial phase of renal I/R injury. Up-regulation of adhesion molecules on endothelial cells, induced by locally produced pro-inflammatory mediators, is amongst the first changes observed after renal I/R injury and is central to the pathogenesis of ischemic acute kidney injury [1,16]. Subsequently leukocytes become activated by local pro-inflammatory factors, thereby facilitating adherence to endothelial cells and subsequent renal tissue infiltration [1]. Sequestered neutrophils induce parenchymal damage, followed by cytokine production by resident renal cells and infiltrating cells, which pro-

motes further tissue damage [1,17]. It has been demonstrated that renal mRNA expression of the early adhesion molecule E-selectin peaks within 6 hours post-reperfusion, with neutrophils infiltrating in parallel. E-selectin blockage with the selectin specific ligand sPSGL has been shown to inhibit renal neutrophil infiltration after I/R and to preserve kidney function [18]. In mice treated with AQGV we observed decreased E-selectin mRNA levels 6 hrs post-reperfusion and decreased renal neutrophil infiltration at 24 hours post-reperfusion. Apoptotic cell death, an important determinant of cellular damage in ischemic kidneys [19] was also significantly reduced in AQGV treated mice. Additionally, serum levels of the inflammatory cytokines TNF-α, INF-γ, IL-6, and IL-10 were significantly decreased 24 hours post-reperfusion upon AQGV treatment. This data is indicative of decreased renal injury and fits with the preservation of kidney function we observed upon AQGV treatment. The lower levels of systemic cytokines observed upon AQGV treatment may be a reflection of reduced formation as well as better renal clearance of these cytokines [20]. The lower serum cytokine levels likely contribute to the decreased mortality by preventing systemic inflammation and subsequent complications in these animals [21].

Our data indicate that AQGV treatment protects against renal I/R injury by interfering with early E-selectin upregulation, thereby reducing neutrophil influx, parenchymal damage and possibly cytokine production. So far it is unclear what the molecular mechanism of action is by which AQGV exerts its effects. It is possible that AQGV mediates its effect by an as yet unidentified receptor. However, we cannot exclude the possibility that, due to the small size and molecular weight, AQGV penetrates the cell membrane [22] and exerts its action either by interfering with signaling cascades or the transcriptional machinery. E-selectin is expressed de novo on endothelial cells after transcriptional induction by pro-inflammatory agents [23]. Whether AQGV inhibits the local production of pro-inflammatory mediators that induce E-selectin or directly interferes with the intracellular signalling cascade involved in activating E-selectin transcription is not clear so far. In contrast to E-selectin, ICAM-1 expression was not altered by peptide treatment. The transcription factor HMGA1 is required for optimal activation of E-selectin gene transcription while it has no role in activating ICAM-1 transcription [24,25] Therefore it is possible that AQGV interferes specifically with pathways required for E-selectin transcriptional activation.

Although previous work revealed a pathophysiologic role of the T-cell as mediator of ischemic ARF [26,27], we did not find a significant difference between the AQGV and placebo treated mice in numbers of CD4+, CD8+ T-cells or macrophages. Our data fit with the observation that RAG-1 deficient mice (lacking both T- and B-cells) are not protected from renal I/R injury [28].

Ki-67, a marker for cellular proliferation, is part of a nuclear protein complex expressed in the G1, S, G2 and M phases of the cell cycle in proliferating cells [29,30]. Mice treated with AQGV showed significantly increased numbers of Ki-67 positive renal tubular epithelial cells at 24 hr post-reperfusion, reflecting enhancement of the regenerative process [31]. The increase in proliferation is likely facilitated by a reduction in inflammation-induced tissue injury, since high levels of pro-inflammatory cytokines have been shown to suppress regeneration of ischemically damaged kidneys [32].

AQGV treatment, at a dose of 5 mg/kg BW, given at either 12 and 24 hr post-reperfusion or at

6 and 12 hr post-reperfusion was not associated with improved survival. Although we cannot formally exclude that these post-reperfusion treatment regimens improved kidney function, it appears that in the currently used model AQGV only prevents the onset of renal ischemia reperfusion injury. This may indicate that AQGV inhibits the activation of pathophysiological pathways involved in renal ischemia reperfusion injury, but is unable to reverse these pathways once activated. However, since we do not exclude that higher doses of AQGV given post-reperfusion do reverse renal-ischemia reperfusion injury, detailed dose-response studies are warranted to gain full insight into the renoprotective effect of AQGV, and other hCG-related oligopeptides.

In conclusion, this study shows that treatment of mice with 5 mg/kg of either one of the hCG-related oligopeptides MTRV, LQG, VLPALPQ or AQGV shortly before and immediately after renal pedicle clamping can significantly reduce mortality and ameliorate kidney injury in a model of warm ischemia reperfusion injury. Of the various oligopeptides evaluated, AQGV appeared to be the most potent one. The renoprotective effect of AQGV was associated with decreased renal E-selectin transcripts, decreased renal neutrophil infiltration, reduced numbers of apoptotic tubular epithelial cells and a reduction of systemic levels of TNF-α, IFN-γ, IL-6 and IL-10. This data implies that AQGV interferes with the early renal inflammatory response induced by I/R and as such prevents parenchymal damage and organ dysfunction. These new renoprotective oligopeptides show great promise for preventing the development of renal ischemia reperfusion injury and may well be used in clinical situations where renal I/R is foreseeable, such as semi-elective surgeries including kidney transplantation, cardiac surgery, and abdominal aorta surgery. So far, phase 1A and phase 1B studies with AQGV (EA-230) have been successfully completed and phase II studies are underway [33,34].

ACKNOWLEDGEMENTS

This study was financially supported by Biotempt B.V., Koekange, The Netherlands.

REFERENCES

1 BONVENTRE JV, WEINBERG JM: RECENT ADVANCES IN THE PATHOPHYSIOLOGY OF ISCHEMIC ACUTE RENAL FAILURE. J AM SOC NEPHROL 2003;14:2199-2210.

2 MUYAN M, BOIME I: SECRETION OF CHORIONIC GONADOTROPIN FROM HUMAN TROPHOBLASTS. PLACENTA 1997;18:237-241.

3 BIRKEN S, MAYDELMAN Y, GAWINOWICZ MA, POUND A, LIU Y, HARTREE AS: ISOLATION AND CHARACTERIZATION OF HUMAN PITUITARY CHORIONIC GONADOTROPIN. ENDOCRINOLOGY 1996;137:1402-1411.

4 YOSHIMOTO Y, WOLFSEN AR, HIROSE F, ODELL WD: HUMAN CHORIONIC GONADOTROPIN--LIKE MATERIAL: PRESENCE IN NORMAL HUMAN TISSUES. AM J OBSTET GYNECOL 1979;134:729-733.

5 COLE LA, KARDANA A, PARK SY, BRAUNSTEIN GD: THE DEACTIVATION OF HCG BY NICKING AND DISSOCIATION. J CLIN ENDOCRINOL METAB 1993;76:704-710.

6 KHAN NA, KHAN A, SAVELKOUL HF, BENNER R: INHIBITION OF DIABETES IN NOD MICE BY HUMAN PREGNANCY FACTOR. HUM IMMUNOL 2001;62:1315-1323.

7 KHAN NA, KHAN A, SAVELKOUL HF, BENNER R: INHIBITION OF SEPTIC SHOCK IN MICE BY AN OLIGOPEPTIDE FROM THE BETA-CHAIN OF HUMAN CHORIONIC GONADOTROPHIN HORMONE. HUM IMMUNOL 2002;63:1-7.

8 BENNER R, KHAN NA: DISSECTION OF SYSTEMS, CELL POPULATIONS AND MOLECULES. SCAND J IMMUNOL 2005;62 SUPPL 1:62-66.

9 ALFTHAN H, STENMAN UH: PATHOPHYSIOLOGICAL IMPORTANCE OF VARIOUS MOLECULAR FORMS OF HUMAN CHORIOGONADOTROPIN. MOL CELL ENDOCRINOL 1996;125:107-120.

10 COLE LA, KARDANA A, ANDRADE-GORDON P, GAWINOWICZ MA, MORRIS JC, BERGERT ER, O'CONNOR J, BIRKEN S: THE HETEROGENEITY OF HUMAN CHORIONIC GONADOTROPIN (HCG). III. THE OCCURRENCE AND BIOLOGICAL AND IMMUNOLOGICAL ACTIVITIES OF NICKED HCG. ENDOCRINOLOGY 1991;129:1559-1567.

11 KARDANA A, ELLIOTT MM, GAWINOWICZ MA, BIRKEN S, COLE LA: THE HETEROGENEITY OF HUMAN CHORIONIC GONADOTROPIN (HCG). I. CHARACTERIZATION OF PEPTIDE HETEROGENEITY IN 13 INDIVIDUAL PREPARATIONS OF HCG. ENDOCRINOLOGY 1991;129:1541-1550.

12 BIRKEN S, MAYDELMAN Y, GAWINOWICZ MA: PREPARATION AND ANALYSIS OF THE COMMON URINARY FORMS OF HUMAN CHORIONIC GONADOTROPIN. METHODS 2000;21:3-14.

13 DIK WA, NADEL B, PRZYBYLSKI GK, ASNAFI V, GRABARCZYK P, NAVARRO JM, VERHAAF B, SCHMIDT CA, MACINTYRE EA, VAN DONGEN JJ, LANGERAK AW: DIFFERENT CHROMOSOMAL BREAKPOINTS IMPACT THE LEVEL OF LMO2 EXPRESSION IN T-ALL. BLOOD 2007;110:388-392.

14 SACKS G, SARGENT I, REDMAN C: AN INNATE VIEW OF HUMAN PREGNANCY. IMMUNOL TODAY 1999;20:114-118.

15 VAN DEN BERG HR, KHAN NA, VAN DER ZEE M, BONTHUIS F, IJZERMANS JN, DIK WA, DE BRUIN RWF: SYNTHETIC OLIGOPEPTIDES RELATED TO THE BETA-SUBUNIT OF HCG REDUCE INFLAMMATION AND LIVER DAMAGE AFTER HEMORRHAGIC SHOCK AND RESUSCITATION. SHOCK, IN PRESS 2008

16 KELLY KJ, WILLIAMS WW, JR., COLVIN RB, MEEHAN SM, SPRINGER TA, GUTIERREZ-RAMOS JC, BONVENTRE JV: INTERCELLULAR ADHESION MOLECULE-1-DEFICIENT MICE ARE PROTECTED AGAINST ISCHEMIC RENAL INJURY. J CLIN INVEST 1996;97:1056-1063.

17 DONNAHOO KK, SHAMES BD, HARKEN AH, MELDRUM DR: REVIEW ARTICLE: THE ROLE OF TUMOR NECROSIS FACTOR IN RENAL ISCHEMIA REPERFUSION INJURY. J UROL 1999;162:196-203.

18 TAKADA M, NADEAU KC, SHAW GD, MARQUETTE KA, TILNEY NL: THE CYTOKINE-ADHESION MOLECULE CASCADE IN ISCHEMIA/REPERFUSION INJURY OF THE RAT KIDNEY. INHIBITION BY A SOLUBLE P-SELECTIN LIGAND. J CLIN INVEST 1997;99:2682-2690.

19 KAUSHAL GP, BASNAKIAN AG, SHAH SV: APOPTOTIC PATHWAYS IN ISCHEMIC ACUTE RENAL FAILURE. KIDNEY INT 2004;66:500-506.

20 BOCCI V, PAULESU L, PESSINA GP: THE RENAL CATABOLIC PATHWAYS OF CYTOKINES. CONTRIB NEPHROL 1993;101:55-60.

21 KELLY KJ: DISTANT EFFECTS OF EXPERIMENTAL RENAL ISCHEMIA/REPERFUSION INJURY. J AM SOC NEPHROL 2003;14:1549-1558.

22 LIPINSKI CA, LOMBARDO F, DOMINY BW, FEENEY PJ: EXPERIMENTAL AND COMPUTATIONAL APPROACHES TO ESTIMATE SOLUBILITY AND PERMEABILITY IN DRUG DISCOVERY AND DEVELOPMENT SETTINGS. ADV DRUG DELIV REV 2001;46:3-26.

23 BEVILACQUA MP, STENGELIN S, GIMBRONE MA, JR., SEED B: ENDOTHELIAL LEUKOCYTE ADHESION MOLECULE 1:

AN INDUCIBLE RECEPTOR FOR NEUTROPHILS RELATED TO COMPLEMENT REGULATORY PROTEINS AND LECTINS. SCIENCE 1989;243:1160-1165.

24 COLLINS T, READ MA, NEISH AS, WHITLEY MZ, THANOS D, MANIATIS T: TRANSCRIPTIONAL REGULATION OF ENDOTHELIAL CELL ADHESION MOLECULES: NF-KAPPA B AND CYTOKINE-INDUCIBLE ENHANCERS. FASEB J 1995;9:899-909.

25 SHANNON MF, COLES LS, ATTEMA J, DIAMOND P: THE ROLE OF ARCHITECTURAL TRANSCRIPTION FACTORS IN CYTOKINE GENE TRANSCRIPTION. J LEUKOC BIOL 2001;69:21-32.

26 BURNE MJ, DANIELS F, EL GHANDOUR A, MAUIYYEDI S, COLVIN RB, O'DONNELL MP, RABB H: IDENTIFICATION OF THE CD4(+) T CELL AS A MAJOR PATHOGENIC FACTOR IN ISCHEMIC ACUTE RENAL FAILURE. J CLIN INVEST 2001;108:1283-1290.

27 TAKADA M, CHANDRAKER A, NADEAU KC, SAYEGH MH, TILNEY NL: THE ROLE OF THE B7 COSTIMULATORY PATHWAY IN EXPERIMENTAL COLD ISCHEMIA/REPERFUSION INJURY. J CLIN INVEST 1997;100:1199-1203.

28 PARK P, HAAS M, CUNNINGHAM PN, BAO L, ALEXANDER JJ, QUIGG RJ: INJURY IN RENAL ISCHEMIA REPERFUSION IS INDEPENDENT FROM IMMUNOGLOBULINS AND T LYMPHOCYTES. AM J PHYSIOL RENAL PHYSIOL 2002;282:F352-357.

29 SCHOLZEN T, GERDES J: THE KI-67 PROTEIN: FROM THE KNOWN AND THE UNKNOWN. J CELL PHYSIOL 2000;182:311-322.

30 RENSHAW AA, LOUGHLIN KR, DUTTA A: CYCLIN A AND MIB1 (KI67) AS MARKERS OF PROLIFERATIVE ACTIVITY IN PRIMARY RENAL NEOPLASMS. MOD PATHOL 1998;11:963-966.

31 COMPERAT E, FERLICOT S, CAMPARO P, ESCHWEGE P, BA N, BENOIT G, BEDOSSA P, PARADIS V: EXPRESSION OF EPIDERMAL GROWTH FACTOR RECEPTOR AND PROLIFERATIVE ACTIVITY OF CYST EPITHELIUM IN HUMAN RENAL CYSTIC DISEASES. UROL INT 2006;76:269-273.

32 VINUESA E, SOLA A, JUNG M, ALFARO V, HOTTER G: LIPOCALIN-2-INDUCED RENAL REGENERATION DEPENDS ON CYTOKINES. AM J PHYSIOL RENAL PHYSIOL 2008;295:F1554-1562.

33 EXPONENTIAL BIOTHERAPIES ANNOUNCES COMPLETION OF EA-230 PHASE 1B SINGLE DOSE HUMAN LPS CHALLENGE, PRESS RELEASE, HTTP//:WWW.EXPOBIO.COM/DOCS/10-20-05.PDF, 2005.

34 EXPONENTIAL BIOTHERAPIES ANNOUNCES COMPLETION OF EA-230 PHASE 1A MULTI-DOSE HUMAN SAFETY TRIALS, PRESS RELEASE, HTTP//:WWW.EXPOBIO.COM/DOCS/3-10-06.PDF, 2006,

35 LAPTHORN AJ, HARRIS DC, LITTLEJOHN A, LUSTBADER JW, CANFIELD RE, MACHIN KJ, MORGAN FJ, ISAACS NW: CRYSTAL STRUCTURE OF HUMAN CHORIONIC GONADOTROPIN. NATURE 1994;369:455-461.

Chapter 7

Donor pre-treatment with tacrolimus reduces transplant vasculopathy

D Susa, S van den Engel, LCA van Damme*, HP Roest, R Krams*, JNM IJzermans, RWF de Bruin

Department of Surgery and Biomedical Engineering*, Thoraxcenter, Erasmus Medical Center, Rotterdam, The Netherlands

Pharmacological Research 2009; 59 (4): 273-8

ABSTRACT

We tested whether transplant arteriosclerosis can be reduced by pre-treatment of the donor with immunosuppressive agents, using a rat allogeneic aorta transplantation model.

Donor rats received no pre-treatment, or tacrolimus, methylprednisolone, rapamycin, or mycofenolate mofetil (MMF) 16 and 2 hours before explantation of the grafts. Eight weeks after transplantation, aorta allografts were harvested. Percent intima area / intima + media area (I/I+M), inflammatory cells and in-situ MMP-2 and -9 activity were determined. In pre-transplantation biopsies, MMP-2 and -9 ratio, and mRNA levels for genes of interest were determined.

In pre-transplantation biopsies we found no differences in MMP-2/9 ratio, and Bcl-2, Bax, TGF-β, HO-1, p21, and HIF-1α mRNA expression between the groups.

Aorta allografts, pre-treated with tacrolimus, showed significantly lower I/I+M ratio compared to untreated controls ($p<0.01$). Pre-treatment with methylprednisolone, rapamycin or MMF did not significantly reduce I/I+M ratio. In-situ MMP-2/ MMP-9 activity was significantly reduced in grafts treated with tacrolimus and rapamycin compared to controls ($p<0.05$). Immunohistochemistry revealed a high number of CD4+ cells and high CD4/CD8 ratio in grafts pre-treated with tacrolimus.

Donor pre-treatment with tacrolimus significantly reduces transplant arteriosclerosis and is associated with reduced in-situ MMP-2 / MMP-9 activity and increased numbers of CD4+ cells.

INTRODUCTION

Although organ transplantation is the treatment of choice for end stage organ failure, two major problems facing the transplant community remain; Long-term survival of organ grafts has still not reached its full potential, and the shortage of suitable donor organs is still increasing [1].
Long-term graft survival is significantly hampered by chronic transplant dysfunction (CTD). This still poorly defined way of untreatable functional deterioration of an organ following transplantation accounts for approximately 30 percent of graft loss in the first 5 years after transplantation. The histomorphological hallmark of CTD in all parenchymal allografts is vasculopathy. This process is characterized by neo-intima formation due to migration and proliferation of vascular smooth muscle cells into the intima [2].
It was recently shown that pre-treatment of kidney allograft donors with dopamine significantly reduces inflammation and tubulitis after transplantation, which may reduce graft damage, and improve long term outcome [3]. In addition, the immunosuppressive drugs cyclosporin [4,5], tacrolimus [4-6], mycophenolate mofetil [7] and methylprednisolone [8,9] have been shown to reduce ischemic injury, which is an inevitable consequence of organ transplantation.
These findings suggest that pre-treatment of organ donors with immunosuppressive agents may result in an improvement in graft function, which may be beneficial to long term graft survival. Thus, commercially used immunosuppressants may not only protect the graft against rejection after transplantation, but may also protect the donor organ against the consequences of preservation, surgery, and immunologically induced injury.
The aim of the present study therefore is to investigate whether donor pre-treatment with immunosuppressive agents reduces transplant vasculopathy using the rat aorta transplantation model.

MATERIALS AND METHODS

Experimental design

The experimental protocol was approved by the Animal Experiment Committee under the national Experiments on Animals Act and adhered to the rules laid down in this national law that serves the implementation of "Guidelines on the protection of experimental animals" by the Council of Europe (1986), Directive 86/609/EC.
Donors were divided in four groups and received commercially available immunosuppression 16 and 2 hours before harvesting of the graft. These time points were chosen since they fit within the time frame between diagnosis of brain death and organ retrieval which is usually 10 - 24 hours in the clinic.
Controls: no pre-treatment, tacrolimus (Prograft, Astellas, the Netherlands): 5 mg kg^{-1} by oral gavage, mycofenolate mofetil (MMF) (CellCept, Roche, the Netherlands): 20 mg kg^{-1} by oral gavage, rapamycin (Rapamune, Wyeth, The Netherlands): 5 mg kg^{-1} by oral gavage, and methylprednisolone (Solu-Medrol, Pharmacia, The Netherlands): 5 mg kg^{-1} intravenously (Table 1). Five to ten animals per group were studied. Immediately after harvesting of the grafts, a tissue sample was snap frozen in liquid nitrogen for future analysis.
Eight weeks after transplantation, graft recipients were killed, and the aorta grafts harvested. In-

timal thickness and vascular remodelling were measured in histological slides. Infiltrating cells were analysed using immunohistochemistry, MMP activity was determined by zymography, and mRNA expression determined by quantitative PCR.

Group	Donor pre-treatment	Dosage
1	None	
2	Tacrolimus	5 mg / kg
3	Mycofenolate mofetil	20 mg / kg
4	Rapamycin	5 mg / kg
5	Methylprednisolone	5 mg/kg

Table 1. Experimental groups
Donors were divided in four groups and received commercially available immunosuppression 16 and 2 hours before harvesting of the graft a follows: controls: no pre-treatment, tacrolimus: 5 mg/kg by oral gavage, mycofenolate mofetil: 20 mg/kg orally, rapamycin: 5 mg/kg orally, methylprednisolone: 5 mg/kg intravenously.

Animals

Male Brown Norway (BN) ($Rt1^n$) and Wistar Augouti (WAG) ($Rt1^u$) rats of 12-14 weeks old, weighing 250–300g, were purchased from Harlan-CPB (Austerlitz, The Netherlands). They were housed three per cage under standard laboratory conditions. The animals had free access to water and rat chow (Hope Farms, Woerden, The Netherlands).

Aorta transplantation

Intra-abdominal aorta transplantation was done in the fully allogeneic BN to WAG rat model. Donor and recipient animals were anesthetized with isoflurane. Anesthesia was maintained using a mixture of N_2O/O_2/isoflurane. A segment of infra-renal aorta of approximately 1cm was isolated, excised, perfused with saline, and stored in 4°C PBS until transplantation, approximately 20 minutes later. In the recipient a similar aorta segment was removed, and the graft was anastomosed end-to-end to the recipient aorta using 9/0 sutures (B. Braun, Melsungen, Germany).

Histology

Aortic grafts were fixed in 3,6% formalin for 24 hours, and embedded in paraffin wax. Five μm sections were cut and stained with haematoxylin-eosin and elastica van Giesson. The area of the intima and area of the media were calculated using a computerized image analysis system (Q-Win, Leica, Germany). Results were expressed as mean ± SD of the intima area divided by the sum of intima and media area) (I/I+M ratio) x 100.

Immunohistochemistry

Frozen sections (5 μm) were cut and stored at –20°C until used. For immunohistochemical detection of graft infiltrating cells, slides were air dried and fixed in acetone for 10 min at room temperature and rinsed with Phosphate Buffered Saline (PBS). Endogenous peroxidase was blocked in a 0,03% H_2O_2 solution. After pre-incubation with 10% Normal Rabbit Serum (DAKO) for 10 min at room temperature in PBS/5% BSA, sections were incubated with mouse-anti-rat primary antibodies against CD4, CD8, CD45, CD5, ED1, or granulocytes (Serotec, The Netherlands) for 30 minutes at room temperature. The slides were rinsed two times with PBS and incubated with a mixture of rabbit-anti-mouse IgG, 2% rat serum and 2% rabbit serum for 30 min at RT. After rinsing the slides two times in PBS they were labelled using PAP complex. Enzyme detection was performed by incubation with DAB for 8 min. Subsequently, the slides were rinsed in tap water, counterstained with hematoxylin, dehydrated in increasing concentrations of alcohol, cleared with xylene and mounted using Pertex. As a negative control the primary antibody was omitted. Positive cells were counted using a Leica DM4000 light microscope using a magnification of 400x, and expressed as total number of cells per mm^2 ± SEM.

RNA and protein isolation

One mm sections of aorta explants were homogenized for 30 seconds using a pro 200 homogenizer equipped with a 02-07075 generator (PRO scientific inc., Oxford, CT) in 0.5 ml Trizol (Invitrogen, Breda, The Netherlands). Total RNA was isolated according to the manufacturer's guidelines with 10 μg RNAse-free glycogen per sample as a carrier (Invitrogen), precipitated and subsequently dissolved in 25 μl DEPC-treated water. RNA concentration was determined using a Nanodrop ND-1000 UV-VIS spectrophotometer (Nanodrop technologies, Wilmington, DE) and stored at -80°C until further use. Proteins were isolated from the phenol-chloroform phase as described in the instructions supplied by the manufacturer, and dissolved in 1% SDS/50 mM NaOH. Samples were stored at -20°C until used.

cDNA synthesis

Approximately 1 μg of total RNA was used as template for first-strand cDNA synthesis using M-MLV Reverse Transcriptase (Invitrogen) according to the protocol of the supplier. 500 ng of oligo$(dT)_{18}$ was used as a starting point for reversed transcription. All temperature-dependent steps were performed using a Biometra T-gradient cycler (Biometra Gmbh, Göttingen, Germany). cDNA samples were stored at -20°C.

Primers

Primers used to amplify the genes of interest can be found in Table 2.

Gene	Forward	Reverse
TGF-β	5-actggagttgtccggcagtgg-3	5-tcacctcgacgtttgggactg-3
Bcl-2	5-acatcgccctgtggatgactg-3	5-gcatgctggggccatatagttc-3
Bax	5-tgctgatggcaacttcaactgg-3	5-tggttctgatcagctcgggca-3
Hif-1α	5-agtcagcaacgtggaaggtgc-3	5-acaaatcagcaccaagcacgtc-3
p21	5-ccacagcgatatcgagacactca-3	5-acagacgacggcatactttgctc-3
HO-1	5-tttcagaagggtcaggtgtcca-3	5-agtagagcggggcatagactgg-3
β-actin	5-gacccagatcatgtttgagacc-3	5-gatgggcacagtgtgggtgac-3

Table 2. Primers used

q-PCR analysis

For quantitative analysis of transcription, 1 µl of diluted cDNA was amplified in an Opticon DNA engine (MJ Research, Waltham, MA). Reaction mixtures of 25 µl contain 1 µl of cDNA, 5 pmol of each primer, 0.5 U Platinum Taq (Invitrogen), 20 mM Tris-HCl (pH 8.4), 50 mM KCL, 1.5 mM $MgCl_{2,}$ 0.2 mM dNTP each, and 0.75 µl of a 1:2000 diluted SYBR Green I solution (Sigma, Zwijndrecht, The Netherlands). Data are expressed as delta Ct compared to β-actin [10].

Gelatin Zymography

Zymography was essentially carry out as described by Hawkes et al [11]. In short, protein lysates were mixed with 4x sample buffer (0.25 M Tris.Cl, pH 6.8; 8% SDS; 0.02% BFB; 40% glycerol), and heated for 3 minutes at 55°C. Proteins were separated on 12% acrylamide gels. SDS was removed by washing the gels in 2.5% ($^{v}/_{v}$) Triton X-100 twice for 15 minutes. Gels were subsequently incubated for 15 minutes in development buffer (50 mM Tris-HCl, pH8.8; 5 mM $CaCl_2$; 0.02% NaN_3) at room temperature followed by an O/N incubation at 37°C in the same buffer. Gels were stained with 0.1% (w/v) coomassie brilliant blue (CBB) in H_2O:Methanol:Acetic Acid (9:9:2) and destained in the same solution without CBB, until bands are clearly visible against the blue-stained background. Destaining was stopped using H_2O.

Quantification

The degree of gelatinase activity was determined after scanning the gels on a Kodak 1D imaging system (Eastman Kodak, Rochester, NY) using Imagequant 5.2 analysis software (Molecular Dynamics, Amersham Biosciences, Roosendaal, The Netherlands).

In situ zymography

Adapted from the method described by Mook et al. [12]. Fresh, unfixed cryosections were air dried for 10 min. DQ gelatine (Molecular Probes, Leiden, The Netherlands) was in 1% low gelling temperature agarose. Sections were incubated with this mixture at room temperature for 2 hours. Fluorescence of FITC was detected with excitation at 460-500 nm and emission at 512-542 nm. Specificity of MMP gelatinolytic activity was determined by blocking MMP activity with 20mM

EDTA, or performing in situ zymography on 4% formaldehyde fixed sections. The results are expressed as surface area of FITC divided by the total intima surface area.

Statistical Analysis

Data were analysed using ANOVA, followed by a Mann-Whitney-U test. Calculations were performed using SPSS v11.0 for Windows. A p value ≤0.05 was considered statistically significant.

RESULTS

Effect of donor pre-treatment on neointima formation

Eight weeks after orthotopic aorta transplantation, significant neointima formation was observed in the control group and the groups pre-treated with methylprednisolone, MMF and rapamycin. In aorta allografts pretreated with tacrolimus, neointima formation was markedly reduced, and architecture of the media was spared, resulting in a significantly reduced I/(I+M) ratio (Figure 1A). Untreated controls had a I/(I+M) ratio of 51.6±5.0%, which was significantly higher than in tacrolimus pre-treated grafts (40.4±3.1%, p<0.01). I/(I+M) ratios of aortic grafts pre-treated with methylprednisolone (49.4±5.4), rapamycin (49.6±7.3), or MMF (49.1± 6.3) were not different from control values (Figure 1B).

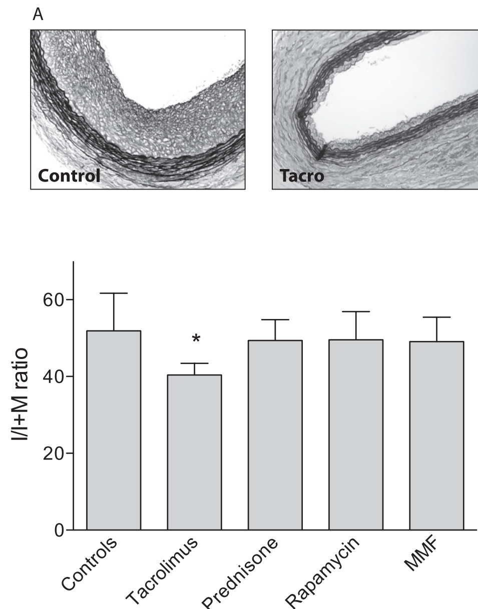

Figure 1. A. Representative histological sections of aorta allografts 8 weeks after transplantation. Significant neointima formation is seen in untreated grafts compared to Tacrolimus pre-treated grafts (elastica van Giesson staining, 100x). B. The area of the intima and area of the media in histological sections was calculated using a computerized image analysis system. Results are expressed as mean ± SD percent intima area / (percent intima area + percent media area) (I/I+M ratio). I/IM ratio in Tacrolimus pre-treated grafts was significantly lower than in untreated controls (*p<0.01).

Effects on cellular infiltration

To study the mechanism underlying the inhibition of intima proliferation after donor-pretreatment with tacrolimus, we investigated cellular infiltration of the grafts. Eight weeks after transplantation, there was a sparse infiltration of cells. Some scattered cells were present in the intima, media and adventitia. Small infiltrates of cells were sometimes observed in the adventitia. The number of CD45+ leukocytes was higher in the tacrolimus group (443 ± 109 cells $mm^{2\ -1}$) compared to untreated controls (231 ± 53 cells mm^{2-1}, p=0.05), and in the MMF- (333 ± 111 cells mm^{2-1}), methylprednisolone- (237 ± 75 cells mm^{2-1}) and rapamycin (225 ± 45 cells mm^{2-1}) pre-treated groups. In the tacrolimus group the increased cell numbers are partially explained by the increased presence of CD5+ T-lymphocytes, but also by the increase in granulocytes (p =0,03 vs. controls). Compared to the other groups, tacrolimus pre-treated animals also showed a remarkably increased number of CD4+ cells (444 ± 155 vs. 71 ± 44 in the control group, p=0.05), and a high CD4/CD8 ratio (2.4 vs 1.2) (Figure 2).

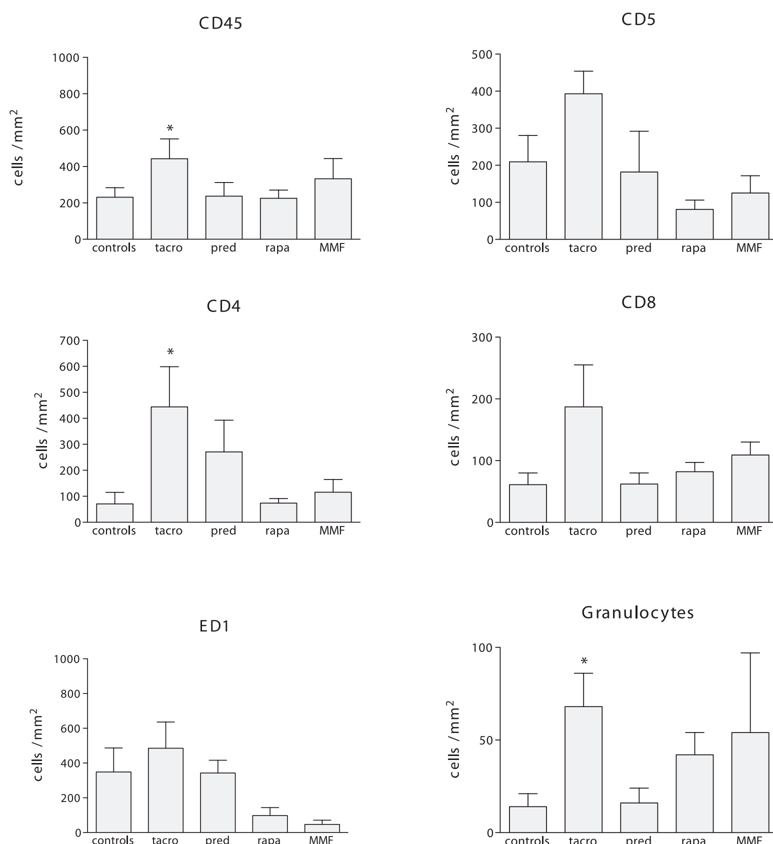

Figure 2. Immunohistochemical analysis of infiltrating cells eight weeks after aorta transplantation. Number of cells ± SEM per mm2 (*p<0.05).

Effect on post-transplantation in situ MMP expression

Using in-situ zymography on fresh cryostat sections we determined the activity of MMP-2 and MMP-9 in the aorta grafts. Activity is expressed as percentage of the total intima. In-situ MMP activity was significantly reduced in grafts treated with tacrolimus (12±0,1%) and rapamycin (11±0,1%) compared to controls (64±3,7%) ($p<0.05$) (Figure 3).
These data were confirmed by gelatin zymography, which showed that the total pro-, and MMP-2 / MMP-9 activity on the gels was reduced by 53% in the tacrolimus pre-treated group (Figure 4).

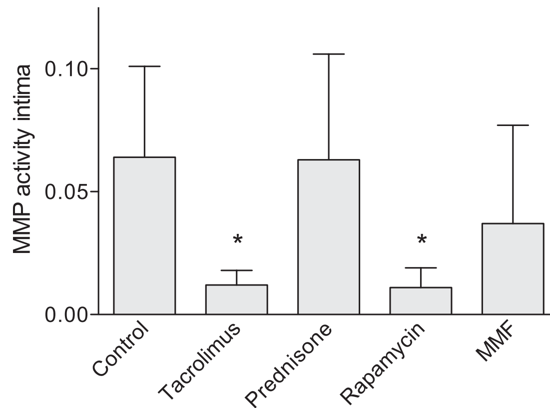

Figure 3. MMP activity as determined by in situ zymography. Results are expressed as mean ± SD total fluorescence per intima (*p<0.05).

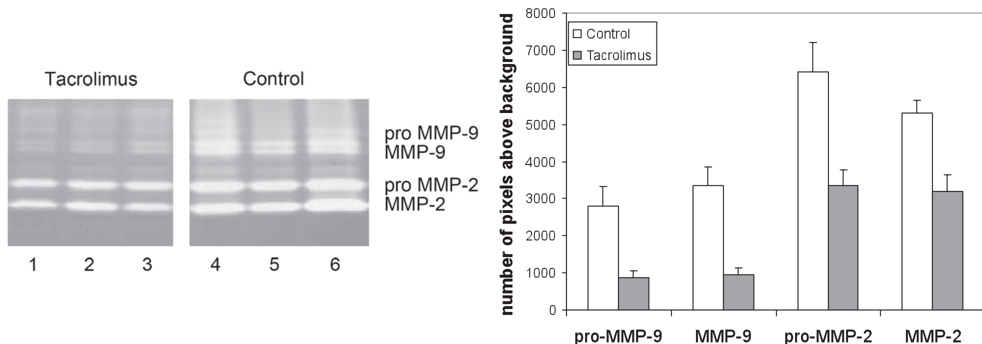

Figure 4. A. zymogram showing reduced MMP-2 and MMP-9 gelatinase activity eight weeks after transplantation in aorta allografts pre-treated with Tacrolimus (1-3) as compared to untreated aorta allografts (4-6). B. quantified gelatinase activity (mean ± SD).

Effect of donor pre-treatment on the pre-transplantation MMP-2/MMP-9 ratio

Donor pre-treatment had no effect on pre-transplant MMP-2 / MMP-9 ratios as measured by gelatin zymography. The ratio was 8±4 in the control group, 10±5 in the Tacrolimus group, 10±4 in the MMF group, and 9±5 in the rapamycin group.

Effect of donor pre-treatment on the pre-transplantation expression of apoptosis and cell-survival related genes

To determine if the beneficial effect of tacrolimus pre-treatment was due to events occurring in the donor, we studied pre-transplantation levels of genes associated with apoptosis, cytoprotection or cell-cycle regulation. We performed real time RT-PCR on cDNA of aorta sections obtained prior to transplantation at the time of harvesting. β-actin was used as house keeping gene. However, BCL-2 and Bax (data not shown), and TGF-β, HO-1, p21, and HIF-1α, levels were not different between the experimental and control groups (Figure 5).

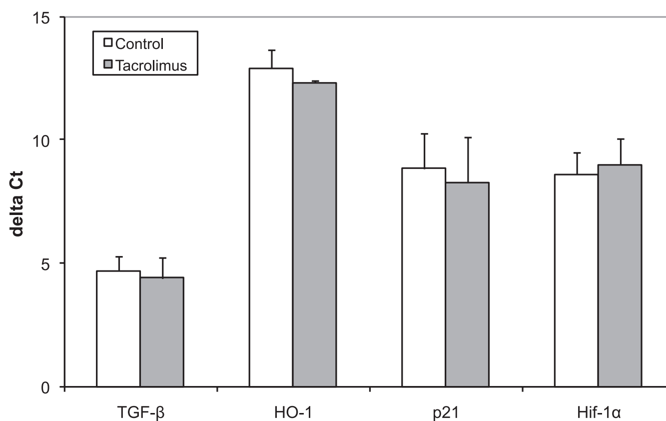

Figure 5. Expression of target genes following pre-treatment of the donor with tacrolimus at 16 and 2 hours before explantation of the graft. mRNA expression is given as delta Ct compared to β-actin (mean ± SD) .

DISCUSSION

We investigated whether pre-treatment of the donor with the immunosuppressive agents tacrolimus, methylprednisolone, rapamycin, and mycophenolate mofetil was able to reduce the development of transplant vasculopathy in a fully allogeneic rat aorta transplantation model. Using this model we have shown previously that transplant vasculopathy develops in a significant and reproducible manner [13], and that this may be reduced by treatment with cyclosporin and ketanserin [14].In the present experimental study, we pretreated transplant donors using a single dosage and treatment schedule. We observed significant neointima formation in the untreated control group eight weeks after orthotopic aorta transplantation, as well as in the methylprednisolone, MMF, and rapamycin pre-treated groups. Acknowledging the limitations of the treatment schedule used, only tacrolimus exerted a significant inhibitory effect on neointima formation in aorta allografts. Although rapamycin treated grafts had significantly lower MMP activity in the neointima, there was no reduction in I/I+M ratio. Future studies should reveal whether this effect can be fortified in a dose-effect relationship, and whether methylprednisolone, MMF, and rapamycin are able to prevent transplant vasculopathy given in other dosages or at different time points.

For now, the mechanisms underlying the protective effect of tacrolimus pre-treatment in our study are highly intriguing. Tacrolimus is able to upregulate TGF-β production, and may stimulate the expression of p21 via a TGF-β dependent pathway [15]. Since p21 is a potent inhibitor of cellular proliferation, upregulation might render the graft in an anti-proliferative state and decrease intima proliferation. TGF-β also induces the cytoprotective protein heme oxygenase-1 (HO-1) [16] which in turn may also upregulate p21 [17]. We speculated that this could be a powerful mechanism protecting the graft against ischemia reperfusion injury and vascular smooth muscle cell proliferation. At the time of harvesting of the graft, however, we did not find a difference in mRNA expression levels of HO-1, TGF-β, p21 or apoptosis related Bcl-2 and Bax. Hence, the protective effect of tacrolimus was not due to pre-transplant induction of cytoprotective or anti-apoptotic genes. Oltean et al. [18] showed that pre-treatment of intestinal grafts with tacrolimus led to blocking of NFκB activation and induction of HSP72 twenty minutes after reperfusion. This effect had disappeared at 6 hours post reperfusion, but resulted in a better microcirculation and morphology and less apoptotic cells in the grafts. The short lived dynamics of these proteins indicate that investigation of additional time points might elucidate the relevance of TGF-β, HO-1 and p21 in our model.

However, using immunohistochemistry, we found increased numbers of CD5+ T-cells after transplantation in grafts from donors pre-treated with tacrolimus, which was caused by an increase in absolute numbers of CD4+ and CD8+ cells as well as an increase in CD4 / CD8 ratio in grafts from donors pre-treated with tacrolimus. This is counterintuitive since most studies show a relation between inhibition of neointima formation and reduced infiltration when immunosuppression is given post-transplantation [19]. These findings suggest that the mechanisms that operate after donor pre-treatment are different from the immunosuppressive mechanisms after recipient treatment. Furthermore, it might suggest a role for a regulatory T cell response in tacrolimus pre-treated grafts. A role for regulatory CD4+ cells in the prevention of chronic allograft dysfunction has been found previously. In a rat kidney transplantation model, Waaga et al. [20] found that T cell clones isolated from animals that displayed tolerance towards the graft produced IL-4 and IL-10 in vivo. Recently, increased numbers of non-regulatory CD4 positive T-cells where shown to infiltrate aorta grafts when aortas were transplanted into CCR7 knockout mice. This was associated with increased transplant arteriosclerosis [21]. The increase in CD4 positive T-cells in our model together with the decreased transplant arteriosclerosis therefore suggests a role for immune regulation by these CD4 positive cells. Our findings corroborate with observations by Reutzel-Selke et al. [22] who found that pre-treatment of rat kidney donors with methylprednisolone and tacrolimus resulted in improved long-term graft outcome. Days after transplantation they found reduced TNF-α, and increased IL-10 levels in the graft. These results also point to an altered inflammatory milieu in the graft and an association between regulatory cytokines and improved long-term graft outcome. Future studies investigating early time-points after aorta transplantation should reveal whether pre-treatment is associated with the induction of Th2 associated cytokines in our model.

Both tacrolimus and rapamycin reduced MMP activity after transplantation, although only tacrolimus reduced neointima formation. It is known that MMP-2 and MMP-9 exert a different function, where MMP-9 is often associated with the formation of neointimal hyperplasia [23,24]. From

these findings, and the associated differences in T-cells we postulate that tacrolimus reduced MMP-9 activity, while rapamycin reduced MMP-2 activity, but studies at earlier time points should reveal their kinetics.Since regulatory T cells produce low levels of MMPs and are not able to stimulate macrophages to produce MMPs, this reduced MMP activity may be mediated by CD4 positive T cells in the graft [25].
In conclusion, this study shows that donor pre-treatment with tacrolimus reduces transplant vasculopathy. The mechanism is suggested to be a T cell related reduction in MMP-9 activity. Further studies to evaluate this mechanism are warranted.

REFERENCES

1 LECHLER RI, SYKES M, THOMSON AW, TURKA LA: ORGAN TRANSPLANTATION--HOW MUCH OF THE PROMISE HAS BEEN REALIZED? NAT MED 2005;11:605-613.

2 MITCHELL RN, LIBBY P: VASCULAR REMODELING IN TRANSPLANT VASCULOPATHY. CIRC RES 2007;100:967-978.

3 LIU Z, HOEGER S, SCHNUELLE P, FENG Y, GOETTMANN U, WALDHERR R, VAN DER WOUDE FJ, YARD B: DONOR DOPAMINE PRETREATMENT INHIBITS TUBULITIS IN RENAL ALLOGRAFTS SUBJECTED TO PROLONGED COLD PRESERVATION. TRANSPLANTATION 2007;83:297-303.

4 SINGH D, CHANDER V, CHOPRA K: CYCLOSPORINE PROTECTS AGAINST ISCHEMIA/REPERFUSION INJURY IN RAT KIDNEYS. TOXICOLOGY 2005;207:339-347.

5 BES S, VANDROUX D, TISSIER C, DEVILLARD L, BROCHOT A, TATOU E, DUVILLARD L, ROCHETTE L, ATHIAS P: DIRECT, PLEIOTROPIC PROTECTIVE EFFECT OF CYCLOSPORIN A AGAINST SIMULATED ISCHEMIA-INDUCED INJURY IN ISOLATED CARDIOMYOCYTES. EUR J PHARMACOL 2005;511:109-120.

6 SAKR M, ZETTI G, MCCLAIN C, GAVALER J, NALESNIK M, TODO S, STARZL T, VAN THIEL D: THE PROTECTIVE EFFECT OF FK506 PRETREATMENT AGAINST RENAL ISCHEMIA/REPERFUSION INJURY IN RATS. TRANSPLANTATION 1992;53:987-991.

7 VENTURA CG, COIMBRA TM, DE CAMPOS SB, DE CASTRO I, YU L, SEGURO AC: MYCOPHENOLATE MOFETIL ATTENUATES RENAL ISCHEMIA/REPERFUSION INJURY. J AM SOC NEPHROL 2002;13:2524-2533.

8 GLANEMANN M, STRENZIOK R, KUNTZE R, MUNCHOW S, DIKOPOULOS N, LIPPEK F, LANGREHR JM, DIETEL M, NEUHAUS P, NUSSLER AK: ISCHEMIC PRECONDITIONING AND METHYLPREDNISOLONE BOTH EQUALLY REDUCE HEPATIC ISCHEMIA/REPERFUSION INJURY. SURGERY 2004;135:203-214.

9 AYDIN G, OKIYE SE, ZINCKE H: A COMPARATIVE STUDY OF SEVERAL AGENTS ALONE AND COMBINED IN PROTECTION OF THE RODENT KIDNEY FROM WARM ISCHAEMIA: METHYLPREDNISOLONE, PROPRANOLOL, FUROSEMIDE, MANNITOL, AND ADENOSINE TRIPHOSPHATE-MAGNESIUM CHLORIDE. UROL RES 1983;11:105-109.

10 PFAFFL MW: A NEW MATHEMATICAL MODEL FOR RELATIVE QUANTIFICATION IN REAL-TIME RT-PCR. NUCLEIC ACIDS RES 2001;29:E45.

11 HAWKES SP, LI H, TANIGUCHI GT: ZYMOGRAPHY AND REVERSE ZYMOGRAPHY FOR DETECTING MMPS, AND TIMPS. METHODS MOL BIOL 2001;151:399-410.

12 MOOK OR, VAN OVERBEEK C, ACKEMA EG, VAN MALDEGEM F, FREDERIKS WM: IN SITU LOCALIZATION OF GELATINOLYTIC ACTIVITY IN THE EXTRACELLULAR MATRIX OF METASTASES OF COLON CANCER IN RAT LIVER USING QUENCHED FLUOROGENIC DQ-GELATIN. J HISTOCHEM CYTOCHEM 2003;51:821-829.

13 GEERLING RA, DE BRUIN RW, SCHERINGA M, BONTHUIS F, JEEKEL J, IJZERMANS JN, MARQUET RL: SUPPRESSION OF ACUTE REJECTION PREVENTS GRAFT ARTERIOSCLEROSIS AFTER ALLOGENEIC AORTA TRANSPLANTATION IN THE RAT. TRANSPLANTATION 1994;58:1258-1263.

14 GEERLING RA, DE BRUIN RW, SCHERINGA M, BONTHUIS F, JN IJ, MARQUET RL: KETANSERIN REDUCES GRAFT ARTERIOSCLEROSIS AFTER ALLOGENEIC AORTA TRANSPLANTATION IN RATS. J CARDIOVASC PHARMACOL 1996;27:307-311.

15 KHANNA AK: THE IMMUNOSUPPRESSIVE AGENT TACROLIMUS INDUCES P21WAF/CIP1WAF1/CIP1 VIA TGF-BETA SECRETION. BIOCHEM BIOPHYS RES COMMUN 2003;303:266-272.

16 NING W, SONG R, LI C, PARK E, MOHSENIN A, CHOI AM, CHOI ME: TGF-BETA1 STIMULATES HO-1 VIA THE P38 MITOGEN-ACTIVATED PROTEIN KINASE IN A549 PULMONARY EPITHELIAL CELLS. AM J PHYSIOL LUNG CELL MOL PHYSIOL 2002;283:L1094-1102.

17 DUCKERS HJ, BOEHM M, TRUE AL, YET SF, SAN H, PARK JL, CLINTON WEBB R, LEE ME, NABEL GJ, NABEL EG: HEME OXYGENASE-1 PROTECTS AGAINST VASCULAR CONSTRICTION AND PROLIFERATION. NAT MED 2001;7:693-698.

18 OLTEAN M, OLOFSSON R, ZHU C, MERA S, BLOMGREN K, OLAUSSON M: FK506 DONOR PRETREATMENT IMPROVES INTESTINAL GRAFT MICROCIRCULATION AND MORPHOLOGY BY CONCURRENT INHIBITION OF EARLY NF-KAPPAB ACTIVATION AND AUGMENTED HSP72 SYNTHESIS. TRANSPLANT PROC 2005;37:1931-1933.

19 GREGORY CR, HUANG X, PRATT RE, DZAU VJ, SHORTHOUSE R, BILLINGHAM ME, MORRIS RE: TREATMENT WITH RAPAMYCIN AND MYCOPHENOLIC ACID REDUCES ARTERIAL INTIMAL THICKENING PRODUCED BY MECHANICAL INJURY AND ALLOWS ENDOTHELIAL REPLACEMENT. TRANSPLANTATION 1995;59:655-661.

20 WAAGA AM, GASSER M, KIST-VAN HOLTHE JE, NAJAFIAN N, MULLER A, VELLA JP, WOMER KL, CHANDRAKER A, KHOURY SJ, SAYEGH MH: REGULATORY FUNCTIONS OF SELF-RESTRICTED MHC CLASS II ALLOPEPTIDE-SPECIFIC TH2 CLONES IN VIVO. J CLIN INVEST 2001;107:909-916.

21 ENSMINGER SM, HELM SN, OHL L, SPRIEWALD BM, ABELE S, WOLLIN M, WOOD KJ, WEYAND M, FORSTER R: INCREASED TRANSPLANT ARTERIOSCLEROSIS IN THE ABSENCE OF CCR7 IS ASSOCIATED WITH REDUCED EXPRESSION OF FOXP3. TRANSPLANTATION 2008;86:590-600.
22 REUTZEL-SELKE A, TULLIUS SG, ZSCHOCKELT T, NIEMINEN-KELHA M, BACHMANN U, JONAS S, PRATSCHKE J, SCHMIDBAUER G, BECHSTEIN WO, VOLK H, NEUHAUS P: DONOR PRETREATMENT OF GRAFTS FROM MARGINAL DONORS IMPROVES LONG-TERM GRAFT OUTCOME. TRANSPLANT PROC 2001;33:970-971.
23 JOHNSON C, GALIS ZS: MATRIX METALLOPROTEINASE-2 AND -9 DIFFERENTIALLY REGULATE SMOOTH MUSCLE CELL MIGRATION AND CELL-MEDIATED COLLAGEN ORGANIZATION. ARTERIOSCLER THROMB VASC BIOL 2004;24:54-60.
24 GALIS ZS, JOHNSON C, GODIN D, MAGID R, SHIPLEY JM, SENIOR RM, IVAN E: TARGETED DISRUPTION OF THE MATRIX METALLOPROTEINASE-9 GENE IMPAIRS SMOOTH MUSCLE CELL MIGRATION AND GEOMETRICAL ARTERIAL REMODELING. CIRC RES 2002;91:852-859.
25 OVIEDO-ORTA E, BERMUDEZ-FAJARDO A, KARANAM S, BENBOW U, NEWBY AC: COMPARISON OF MMP-2 AND MMP-9 SECRETION FROM T HELPER 0, 1 AND 2 LYMPHOCYTES ALONE AND IN COCULTURE WITH MACROPHAGES. IMMUNOLOGY 2008;124:42-50.

Chapter 8

Differential expression of inflammatory, cytoprotective and injury genes in kidneys of heart beating and non heart beating donors

D Susa, HP Roest, NFM Kok, S van den Engel, E Torabi-Azhandeh, JNM IJzermans, RWF de Bruin

Department of Surgery, Laboratory for Experimental Surgery, Erasmus University Medical Centre, Rotterdam, The Netherlands

Submitted

ABSTRACT

The superior long-term survival of kidneys from living donors (LD) compared to brain death (BD) and (NHB) donors is now well established. Comparative studies on transcriptional changes that occur after organ retrieval and cold storage in kidneys from NHB, BD and LD donors are sparse. We examined expression levels of genes indicative of inflammation, cytoprotection, and injury after clinically relevant cold ischemia times. The LD group was analyzed after 2 hours of cold ischemia, the BD and NHB groups after 18 hours. We found massive up-regulation of IL-1β, IL-6, TNF-α, MCP-1, P-selectin, E-selectin, HO-1, p21 and Kim-1 in BD donor kidneys compared to both LD and NHB. Next, we sought if expression levels of these genes would change between explantation of the kidney and the end of the cold storage period. At the time of explantation gene expression levels reflected those after cold storage. Thus, during 18 hours of cold ischemia, these expression levels did not change. We conclude that the gene expression profile of BD kidneys represents an inflammatory and injury response to brain death. In contrast, kidneys from NHB donors show only mild inflammation and injury, compared to kidneys from LD. These results may explain why delayed graft function in NHB kidneys has no deleterious effect on graft survival, as it has in kidneys from BD donors, and lends support to the anti-inflammatory treatment of brain death donors.

INTRODUCTION

Brain death is a risk factor for kidney function and survival following kidney transplantation. Nevertheless, most donor organs are retrieved from brain dead (BD) patients. Improved technology and an increasing gap between supply and demand for donor organs have facilitated an increased use of organs from both living donors (LD) and non heart beating donors (NHB) [1]. Superior long-term survival for LD compared to BD or NHB donors is now well established [2-4]. Living donors are healthy during organ procurement, and retrieved kidneys experience minimal warm and cold ischemia. During the process of brain death in heart beating donors, hemodynamic instability, hormone dysregulation and an immunologic reactivity contribute to organ damage and inferior post-transplant function and graft survival compared to living donors [1, 5, 6]. Organs from NHB donors do not develop the physiological changes of BD donors, but are exposed to prolonged warm ischemia, which increases the incidence of delayed graft function. In addition, organs from both post-mortem groups are exposed to prolonged cold ischemia times compared to living donors, and long term graft survival is comparable between the two [7, 8].

The pathophysiology that leads to organ damage following brain death is relatively well established [5, 9-11], and strategies to protect the brain death donor are emerging [12, 13]. In contrast, experimental studies on the changes that occur in organs from NHB donors are sparse [14].

A drawback of clinical studies comparing peri-operative gene expression patterns during kidney transplantation is that they are confined to a relatively small time span, and a limited number of time points. Usually "t=0" biopsies are obtained at the end of the cold storage period, followed by a post-reperfusion biopsy 30-60 minutes after reperfusion of the graft. These time-points are unable to take into account the major pathophysiological perturbances that occur during BD, cardiac arrest, organ retrieval and the subsequent cold storage period. Moreover, studies may lump together BD and NHB as "cadaveric" donors, making it impossible to discriminate these two groups [15]. Therefore, t=0 biopsies may be of limited value in comparing the changes that occur in BD and NHB donors until explantation of the graft, and the subsequent alterations during the cold storage period. Experimental studies have tried to overcome these limits by studying gene expression at explantation, but these studies have mainly focused on BD donors. Comparative studies in organs from BD, NHB and living donors are currently lacking.

The aim of this study therefore was to compare expression levels of genes indicative of inflammation, cytoprotection, and injury after clinically relevant cold ischemia times, at the time of explantation of kidney grafts from these donor sources as well as over a time-course during cold storage.

RESULTS

Renal gene expression levels after clinically relevant cold ischemia times

mRNA expression levels in BD and NHB and LD kidneys were examined by qRT-PCR after preservation times relevant to human kidney transplantation, namely the end of the cold ischemia period when the transplant is placed in the iliac fossa of the recipient. The LD group was analyzed after 2 hours of cold ischemia (CI), and the BD and NHB groups after 18 hours CI.

Inflammatory genes

Intrarenal IL-1β mRNA levels were 43-fold increased in BD compared to LD donors ($p<0.01$), and 2,3-fold increased in NHB compared to LD ($p<0.01$) (Figure 1A). IL-6 expression was also higher in both BD and NHB, 997- fold ($p<0,01$) and 10-fold ($p<0.01$), respectively, compared to LD. TNF-α levels were 9,7-fold increased in the BD group compared to LD ($p<0.01$). No significant difference in expression was found in NHB kidneys. Monocyte chemotactic protein (MCP-1), was 115-fold increased in BD in comparison to LD ($p<0.01$); no difference was found between LD and NHB. P-selectin was significantly increased in BD 129- fold ($p<0.01$) and NHB (2,9-fold, $p<0.01$). E-selectin was increased in BD by 64-fold ($p<0.01$). Interestingly E-selectin was down-regulated in NHB 0,3-fold compared to LD ($p<0.01$).

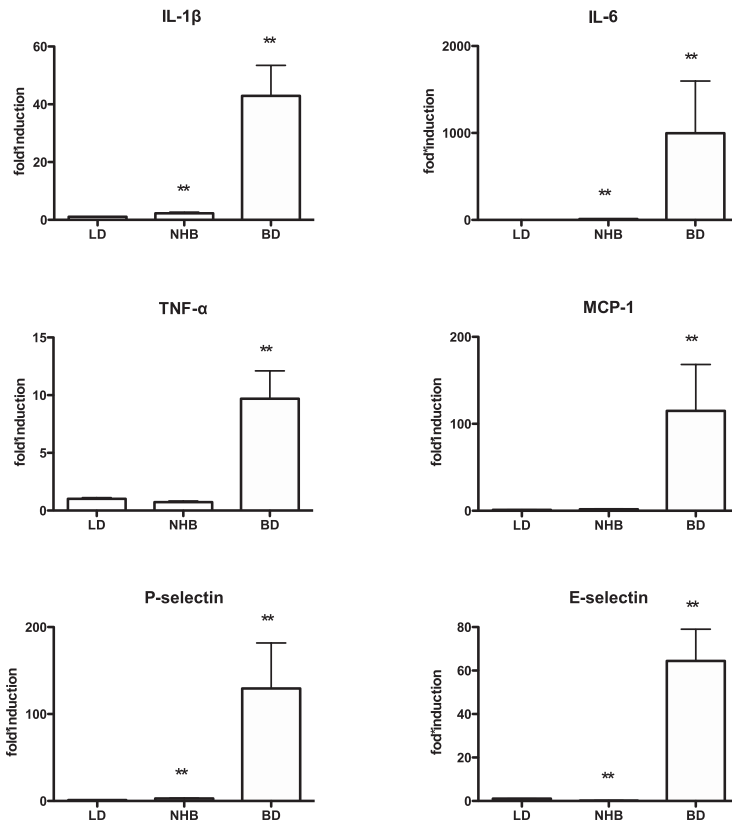

Figure 1A. Gene expression profiles of inflammatory genes in kidneys after clinically relevant cold ischemia times. mRNA expression levels of genes indicative of inflammation were determined by qRT-PCR after 2 hours of cold ischemia in living donors (LD), and after 18 hours of cold ischemia in non-heart beating (NHB) and brain death donors (BD). To compare the individual expression levels of each animal, ΔCt values were normalized to the average ΔCt of the LD group at 2 hours. Subsequently the fold increase was calculated using $2^{\Delta\Delta Ct}$. Results are expressed as mean ± SEM of seven animals per group. *$p<0.05$, **$p<0.01$ compared to LD.

Cytoprotection and injury

After 6 hours of BD, HO-1 was 21-fold up-regulated compared to the LD group ($p<0.01$). In the NHB group HO-1 expression was 0.6-fold lower compared to the control group ($p<0.01$) (Figure 1B). Neither HIF-1α, nor VEGF expression was significantly influenced by BD or NHB compared to LD. The anti-apoptotic gene Bax was expressed higher in the NHB group compared to both LD (1,3-fold, $p<0,05$) but not in the BD group. Expression of Bcl-2 was lower in BD by 0.63-fold compared to LD ($p<0.05$).

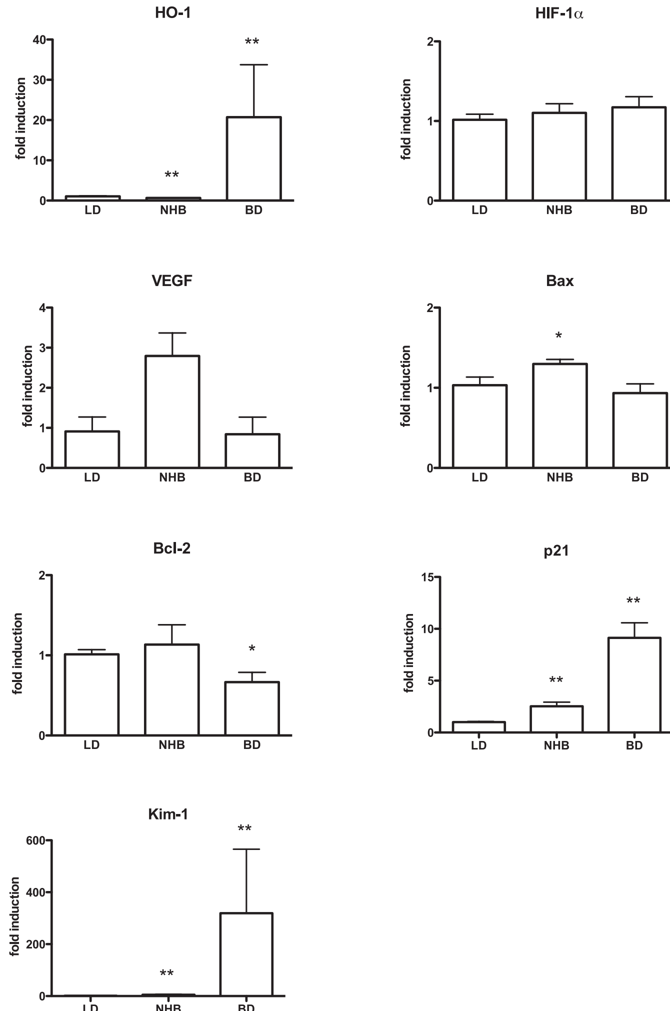

Figure 1B. Gene expression profiles of cytoprotective and injury genes in kidneys after clinically relevant cold ischemia times. mRNA expression levels of genes indicative of inflammation were determined by qRT-PCR after 2 hours of cold ischemia in living donors (LD), and after 18 hours of cold ischemia in non-heart beating (NHB) and brain death donors (BD). To compare the individual expression levels of each animal, ΔCt values were normalized to the average ΔCt of the LD group at 2 hours. Subsequently the fold increase was calculated using $2^{\Delta\Delta Ct}$. Results are expressed as mean ± SEM of seven animals per group. *p<0.05, **p<0.01 compared to LD.

Expression of the cell-cycle inhibitor p21 was higher in both BD and NHB, 9,1- ($p<0.01$) and 2,5-fold ($p<0.01$) respectively as compared to LD. The profound increase in proinflammatory genes in the BD group was accompanied by a strong increase in the expression of Kim-1. In BD, Kim-1 expression was increased 319-fold ($p<0.01$), whereas in NHB kidneys it was increased 5,3-fold ($p<0.01$).

Gene expression levels at explantation of the graft reflect those after cold storage

Next, we sought if expression levels of these genes would change between the moment of explantation of the kidney and the end of the cold storage period. Therefore, gene expression of all groups at the time of explantation (t=0) was analyzed. The obtained expression profiles were comparable to those found at the end of the cold storage period in the previous experiment.

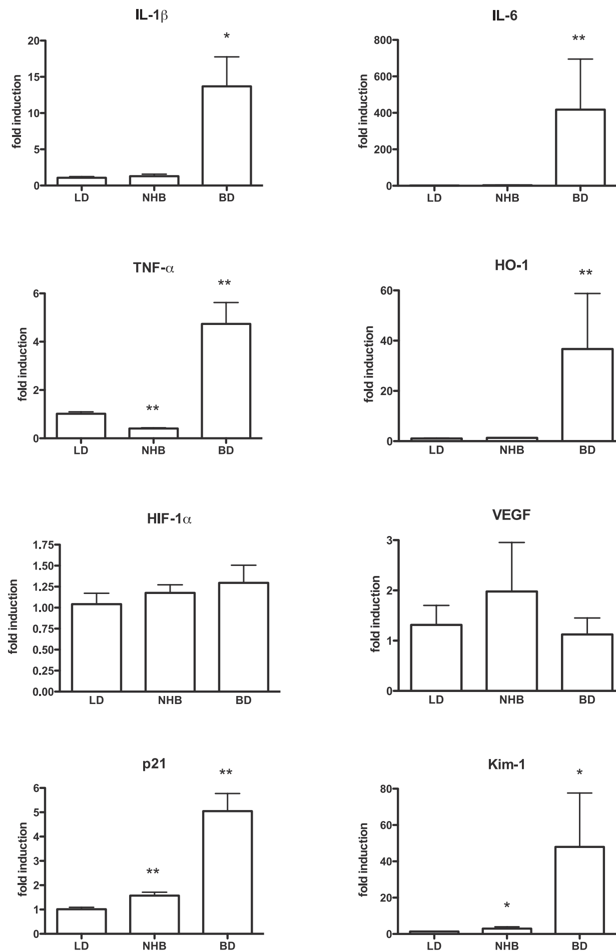

Figure 2. Gene expression levels at explantation of the graft reflect those after cold storage. mRNA expression levels of genes indicative of inflammation, cytoprotection, and kidney injury were determined by qRT-PCR directly after retrieval of the kidneys in living donors (LD), non-heart beating (NHB,) and brain death donors (BD). To compare the individual expression levels of each animal, ΔCt values were normalized to the average ΔCt of the LD group at 2 hours. Subsequently the fold increase was calculated using $2^{\Delta\Delta Ct}$. Results are expressed as mean ± SEM of seven animals per group.

Again, pro-inflammatory genes showed a significant increase in kidneys retrieved from BD animals compared to LD (Figure 2); IL-1β a 13,7-fold, IL-6 a 417-fold, TNF-α a 4,7-fold, and MCP-1 (not shown) a 81-fold increase. In kidneys from NHB animals MCP-1, IL-1b and IL-6 were not significantly different compared to controls. Adhesion molecules P-selectin and E-selectin were both highly elevated in kidneys from BD animals, but not from NHB animals (data not shown).

HO-1 expression was already highly up regulated in BD compared to LD, whereas no difference was found between the NHB and LD group. VEGF expression seemed be up-regulated in NHB, but this was not significant. Similarly, no difference in expression of HIF-1α, Bax (not shown) and Bcl-2 (not shown) was found between the groups at kidney explantation. p21 was significantly elevated in both BD and NHB kidneys ($p<0.01$). These changes were already accompanied by a significant upregulation of Kim-1 expression in both BD (48-fold) and NHB (3-fold).

Gene expression profiles remain stable during an 18 hour cold storage period

To address whether any significant changes in gene-expression would occur following retrieval of the kidneys, gene expression of HO-1, HIF-1α, Bcl-2, Bax, and VEGF (not shown) were examined after 0, 2, 4, 6, 12,and 18 hours of cold storage (Figure 3).

These data confirm the previous findings in this study and show that the donor is the main determinant of gene expression, and that gene expression levels do not significantly alter after retrieval and cold storage of the kidney grafts.

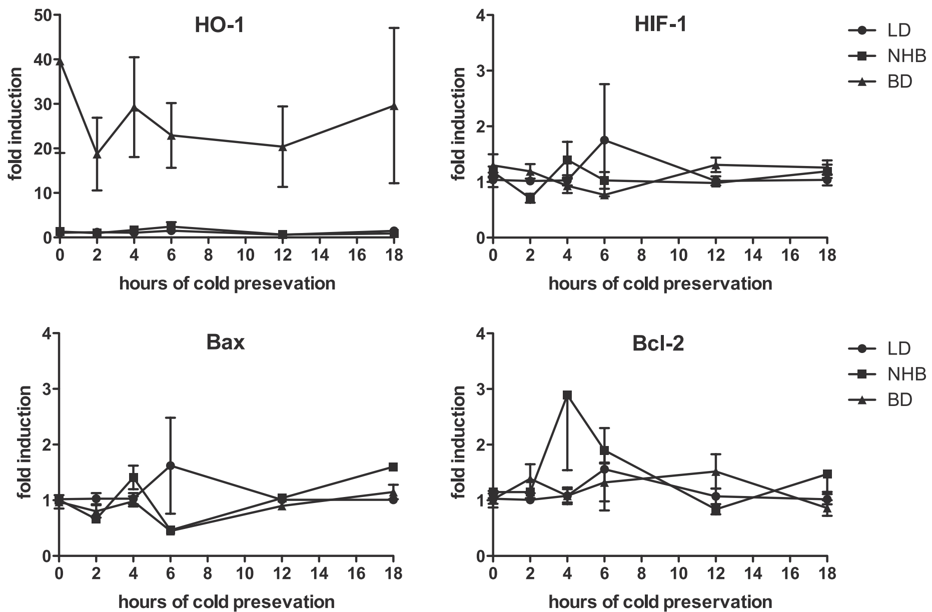

Figure 3. Gene expression profiles in kidneys remain stable during cold preservation. mRNA expression levels of HO-1, HIF-1α, Bax, and Bcl-2 were determined directly after retrieval of kidneys from the donor, and at 2, 4, 6, 12, and 18 hours of cold preservation in kidneys of living donors (LD), non-heart beating (NHB,) and brain death donors (BD). To compare the individual expression levels of each animal, ΔCt values were normalized to the average ΔCt of the LD group at the respective time points. Subsequently the fold increase was calculated using $2^{\Delta\Delta Ct}$. Results are expressed as mean ± SEM of seven animals per group

DISCUSSION

In most countries, the main source of kidney transplants is brain dead patients, although an increasing use of kidneys from living donations and non-heart-beating donors is being reported [16, 17]. Superior long-term results are found in the LD group, whereas organs from brain dead as well as NHB donors show decreased post-transplant function and graft survival [2, 5, 6]. Organs from NHB donors are exposed to prolonged warm ischemia times, and organs from both post-mortem sources are exposed to prolonged cold ischemia times. Delayed graft function is seen more in kidneys from NHB donors compared to BD donors, although long term graft survival is comparable between the two [7, 8]. Studies that have investigated differences in gene expression between these different donors usually use kidney biopsies taken during organ retrieval, after cold ischemia, or after implantation and subsequent reperfusion. Comparison of these groups is difficult since major differences in the course to organ retrieval exist. Therefore, the aim of our study was to compare the impact of brain dead and asystole to living donation, by obtaining kidney biopsy specimens at the moment of organ retrieval, and at the end of cold ischemia. Furthermore, we assessed the effect of an increasing cold preservation period on gene-expression levels. To better understand the relative contributions of brain death and prolonged warm ischemia in BD and NHB donors we studied expression profiles of genes indicative of inflammation, cytoprotection, and kidney injury.

We show that the pathophysiological perturbations induced by brain death are the main determinant of the changes in gene expression, and that mRNA levels do not change after retrieval and cold storage of the graft. Although the prolonged warm ischemia encountered by organs from NHB donors is thought to underlie the increased incidence of delayed graft function (DGF) in these grafts, the deterioration induced by warm ischemia is not reflected by changes in gene expression.

The most striking finding in our study is the difference between brain death and NHB in renal transcription of inflammatory markers such as IL-1β, IL-6, MCP-1, and E-selectin. High levels of inflammatory cytokines in the donor are associated with decreased graft survival [18-20]. The high serum level of IL-6 in serum of BD donors is thought to be due to production in the injured brain and subsequent leakage through the disrupted blood-brain barrier [9, 21]. We show that it is likely that the kidney itself significantly contributes to systemic IL-6 levels, and systemic inflammation observed during brain death.

This inflammation resulted in significant injury of the kidney, as shown by the up-regulation of Kim-1. This gene has been recently identified as a discriminating marker between stable and unstable brain death [10], and as predictor of graft function after BD induced kidney injury [22].

In kidneys from NHB donors, expression of IL-1β, IL-6 and P-selectin was significantly up-regulated compared to LD but to a much lesser extent than in BD donors.

Several factors may contribute to the difference in pro-inflammatory state between BD and NHB. Previous studies [20, 23-25] as well as our own find that a massive inflammatory response develops after brain death. In comparison to this inflammatory response induced during 6 hours of BD, twenty minutes of warm ischemia encountered during non heart beating donation induced only mild changes, which are mostly indistinguishable from the transcriptional changes found after

living donation as presented in this manuscript. In a previous study we have shown that the prolonged warm ischemia encountered during live kidney donation has no functional or histological consequences up to one year after kidney transplantation [26]. When warm ischemic times are prolonged, expression levels may be increased. After a mean long warm ischemic time of 107 minutes in NHB donors, expression of ICAM-1 and VCAM-1 was similarly significantly elevated in BD and NHB kidneys compared to controls [27]. Since the duration of the asystolic period in NHB donors determines the severity of the damage to the kidney [14], it is likely that 20 minutes of asystole used in our study are not enough to induce massive up-regulation of pro-inflammatory gene expression.

The results after NHB donation clearly lag behind those of living related transplantation [2, 3]. The approximately 15 minutes longer ischemia time in NHB compared to LD has no significant effect on transcription, but induces detrimental changes such as ATP loss, Na^+/K^+ misbalance and cell swelling [28]. These differences between NHB and BD are interesting in the light of clinical outcomes after transplantation. Although the incidence of DGF may be almost doubled in NHB donors [7, 8, 29], long-term graft survival is similar in both groups [7, 29]. Thus, although DGF is a risk factor for graft loss in BD donors, it has no effect on graft survival in NHB donors. It has even been suggested that DGF in NHB donors predisposes for favourable graft outcome [30]. The absence of overt inflammatory changes and significantly lower Kim-1 expression in our study may explain why the impact of DGF is reduced in kidneys of NHB donors.

We found a significant up-regulation of HO-1 mRNA in BD donors at the time of explantation, which remained stable during the 18 hour observation period. A body of evidence shows that over-expression of HO-1 and VEGF protect kidney transplants against the deleterious consequences of ischemic injury [31-33]. Both HIF-1α, the transcription factor inducing the production of VEGF, and VEGF itself were not significantly up-regulated in BD donors. This implies that during six hours of BD, the inflammatory response coincides with up-regulation of HO-1, not VEGF. Interestingly, the cyclin-dependant kinase inhibitor p21 was also strongly induced both in NHB and BD donors. p21 which binds stoichiometrically to PCNA and inhibits its action in DNA replication, is a key regulator of proliferation following renal ischemic injury. Mice lacking p21 display hyperproliferation and greatly reduced survival following renal ischemia [34]. We previously showed that there is a critical temporal balance between the expression levels of p21 and proliferation in the regenerating tubular compartment following ischemia [35]. The up-regulation of p21, like HO-1, in our models may be envisioned as indicator of damage, and the lower induction in NHB kidneys may give an advantage following transplantation, when proliferation of the damaged tubular compartment is warranted [34, 35].

The pro-apoptotic gene Bax was slightly, but significantly elevated in NHB, while expression in BD was not different compared to LD. Expression of the anti-apoptotic gene Bcl-2 was down-regulated in BD compared to LD, while expression in NHB was not different. These results suggest that induction of apoptosis may be due to Bax induction during cardiac death, whereas down regulation of Bcl-2 may be responsible in BD donors.

Our study further shows that the expression levels of inflammatory genes encountered at explantation remain stable during an 18 hour cold preservation period.

In conclusion, we show a massive up-regulation of inflammatory, injury, and cytoprotective genes in kidney transplants from BD-, but not living-, or NHB donors at the time of graft explantation. This difference may underlie the observed better graft survival of kidneys from NHB donors [36], and lends further support to the anti-inflammatory treatment of BD donors [12, 13].

MATERIALS AND METHODS

Experimental design

The experimental protocol was approved by the Animal Experiments Committee under the Dutch National Experiments on Animals Act and complied with the 1986 directive 86/609/EC of the Council of Europe. Male Brown Norway (BN) rats of 12-14 weeks old, weighing 250–300g, were purchased from Harlan-CPB (Austerlitz, The Netherlands). Rats were randomly assigned to a living donor (LD), brain death (BD) or non-heart-beating (NHB) group (n=7 per group). The living group served as control for both post-mortem groups. After explantation of the kidneys, cortical kidney samples of all groups were stored in University of Wisconsin (UW) solution. At 0, 2, 4, 6, 12, 18 and 24 hours they were collected and snap frozen in liquid nitrogen until further use.

Experimental models

Animals were anesthetized with isoflurane. After intubation, anesthesia was maintained using a mixture of N_2O/O_2/2%isoflurane. Animals were pressure control ventilated on a Siemens Servo 900C ventilator (Maqet Critical Care AB, Solna, Sweden) with 14 cm H_2O PIP, 4 cm H_2O PEEP with a frequency of 40 breaths per minute.

In the BD group a frontolateral trepanation was made and a balloon catheter (Fogarty Arterial Embolectomy Catheter: 5F, Baxter Healthcare Co., Irvine, Ca, USA) was introduced in the extradural space and slowly inflated, causing a gradually increasing intra-cranial pressure. Herniation of the brainstem and BD was confirmed by dilated and fixed pupils, the absence of corneal reflexes, and an apnoea test. Brain dead animals received ventilation but no further anaesthesia. Intra-arterial pressure was continuously monitored via a PE50 catheter placed in the carotid artery. Only rats with stable mean arterial pressure (MAP > 80 mm Hg) during the 6 hour brain death period were included in the experiment. After 6 hours both kidneys were removed, flushed with PBS and stored in UW solution at 4°C.

In the NHB group the animals were catheterised using a PE50 catheter placed in the carotid artery, and both kidneys were exposed via a midline incision that was then temporarily closed with a few sutures until retrieval of the kidneys. Cardiac arrest was induced by isoflurane overdose. The non heart beating period lasted 20 minutes, starting when the blood pressure had dropped to 5-7 mm Hg. the time commonly needed before starting with cold storage of NHB donor kidneys.

In the living donor group, nephrectomy was performed after one hour of mechanical ventilation using an N_2O/O_2/2% isoflurane mixture.

RNA isolation

Kidney sections were homogenized for 30 seconds using a pro 200 homogenizer equipped with a 02-07075 generator (PRO scientific inc., Oxford, CT) in 1.0 ml Trizol (Invitrogen, Breda, The Netherlands). Total RNA was isolated according to the manufacturer's guidelines, precipitated, and subsequently dissolved in DEPC-treated water. RNA concentration was determined using a Nanodrop ND-1000 UV-VIS spectrophotometer (Nanodrop technologies, Wilmington, DE) and stored at -80°C until further use.

cDNA synthesis

Approximately 1 μg of total RNA was used as template for first-strand cDNA synthesis using M-MLV Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol with 250 ng of random hexamers (Promega, Leiden, The Netherlands) as a starting point for reversed transcription and 40 IU RNAseOUT (Invitrogen) to prevent degradation. All temperature-dependent steps were performed using a Biometra T-gradient cycler (Biometra Gmbh, Göttingen, Germany). cDNA samples were stored at -20°C.

Primers

Primers used to amplify the genes of interest are shown in Table 1.

Gene	Foreward Primer	Reverse Primer
HO-1	'5-tttcagaagggtcaggtgtcca-3'	'5-agtagagcggggcatagactgg-3'
VEGF	'5-tgcactggaccctggctttac-3'	'5-ttctgctccccttctgtcgtg-3'
HIF-1α	'5-agtcagcaacgtggaaggtgc-3'	'5-acaaatcagcaccaagcacgtc-3'
BCl-2	'5-acatcgccctgtggatgactg-3'	'5-gcatgctggggccatatagttc-3'
Bax	'5-tgctgatggcaacttcaactgg-3'	'5-tggttctgatcagctcgggca-3'
IL-1β	'5-gaggctgacagaccccaaaaga-3'	'5-tccacagccacaatgagtgaca-3'
TNF-α	'5-gaccctcacactcagatcatcttct-3'	'5-tgctacgacgtgggctacg-3'
IL-6	'5-gtctcgagcccaccaggaac-3'	'5-agggaaggcagtggctgtca-3'
MCP-1	'5-gccatcagcccacaggtgtt-3'	'5-gggacactggctgcttgtga-3'
P-selectin	'5-aagatggtcagcgcctccac-3'	'5-atcgaaccgatgggacagga-3'
E-selectin	'5-cccacatgtgcaggggtaca-3'	'5-tggcccactgcaactcatgt-3'
Kim-1	'5-ctgcagactggaatggcactgt-3'	'5-gtttctctgcggcttcctcaaa-3'
P21	'5-ccacagcgatatcgagacactca-3'	'5-acagacgacggcatactttgctc-3'
β-actin	'5-gacccagatcatgtttgagacc-3'	'5-gatgggcacagtgtgggtgac-3'

Table 1. Primers used

qRT-PCR analysis

For quantitative analysis of transcription, 0.33 µl of cDNA was amplified using a BioRad Icycler (Bio-Rad laboratories, Veenendaal, The Netherlands). Reaction mixtures of 25 µl contain cDNA, 5

pmol of each primer, 0.5 U Platinum Taq (Invitrogen), 1.5 mM MgCl2, 0.2 mM dNTP each, and 0.075 µl of a 200x stock SYBR Green I solution (Sigma, Zwijndrecht, The Netherlands). Cycling conditions consisted of 3 minutes activation at 95°C, 40 cycles of 15″ 95°C, 30″ 60°C, and 30″ 72°C, followed by 1′ 95°C and 1′ 65°C. Samples were subsequently subjected to a melt curve analysis with a temperature range of 60-94.5°C to verify amplified products.

ΔCt values of genes of interest were calculated as described by Pfaffl et al. using β-actin as a reference gene. To compare all individual expression levels of each animal, ΔCt values were normalized to the average ΔCt of the LD group. The LD group at 2 hours was used in the 2 hour versus 18 hour comparison (Figure 1), the 0 hour group in the the t=0 comparison (Figure 2), and the respective time points in the time course (Figure 3). Subsequently the fold increase was calculated using the Pfaffl equation: $2^{\Delta\Delta Ct}$ [37]. Results are expressed as mean ± SEM.

Statistical Analysis

Data were analysed using a Kruskal Wallis ANOVA, followed by a Mann-Whitney–U test. Analysis was performed using SPSS v11.0 for Windows. A p value <0.05 was considered statistically significant.

REFERENCES

1. SANCHEZ-FRUCTUOSO AI, PRATS D, TORRENTE J, ET AL.: RENAL TRANSPLANTATION FROM NON-HEART BEATING DONORS: A PROMISING ALTERNATIVE TO ENLARGE THE DONOR POOL. J AM SOC NEPHROL 11:350-358, 2000
2. TERASAKI PI, CECKA JM, GJERTSON DW, ET AL.: HIGH SURVIVAL RATES OF KIDNEY TRANSPLANTS FROM SPOUSAL AND LIVING UNRELATED DONORS. N ENGL J MED 333:333-336, 1995
3. CHKHOTUA AB, KLEIN T, SHABTAI E, ET AL.: KIDNEY TRANSPLANTATION FROM LIVING-UNRELATED DONORS: COMPARISON OF OUTCOME WITH LIVING-RELATED AND CADAVERIC TRANSPLANTS UNDER CURRENT IMMUNOSUPPRESSIVE PROTOCOLS. UROLOGY 62:1002-1006, 2003
4. GJERTSON DW, CECKA JM: LIVING UNRELATED DONOR KIDNEY TRANSPLANTATION. KIDNEY INT 58:491-499, 2000
5. SCHUURS TA, MORARIU AM, OTTENS PJ, ET AL.: TIME-DEPENDENT CHANGES IN DONOR BRAIN DEATH RELATED PROCESSES. AM J TRANSPLANT 6:2903-2911, 2006
6. SUSA D, DE BRUIN RWF, MITCHELL JR, ROEST HP, HOEIJMAKERS JHJ, IJZERMANS JNM: MECHANISMS OF AGEING IN CHRONIC ALLOGRAFT NEPHROPATHY. J ORG DYSF 3:7 -17, 2007
7. WEBER M, DINDO D, DEMARTINES N, ET AL.: KIDNEY TRANSPLANTATION FROM DONORS WITHOUT A HEARTBEAT. N ENGL J MED 347:248-255, 2002
8. WIJNEN RM, BOOSTER MH, STUBENITSKY BM, ET AL.: OUTCOME OF TRANSPLANTATION OF NON-HEART-BEATING DONOR KIDNEYS. LANCET 345:1067-1070, 1995
9. BOUMA HR, PLOEG RJ, SCHUURS TA: SIGNAL TRANSDUCTION PATHWAYS INVOLVED IN BRAIN DEATH-INDUCED RENAL INJURY. AM J TRANSPLANT 9:989-997, 2009
10. SCHUURS TA, GERBENS F, VAN DER HOEVEN JA, ET AL.: DISTINCT TRANSCRIPTIONAL CHANGES IN DONOR KIDNEYS UPON BRAIN DEATH INDUCTION IN RATS: INSIGHTS IN THE PROCESSES OF BRAIN DEATH. AM J TRANSPLANT 4:1972-1981, 2004
11. KUSAKA M, KUROYANAGI Y, KOWA H, ET AL.: GENOMEWIDE EXPRESSION PROFILES OF RAT MODEL RENAL ISOGRAFTS FROM BRAIN DEAD DONORS. TRANSPLANTATION 83:62-70, 2007
12. KOTSCH K, ULRICH F, REUTZEL-SELKE A, ET AL.: METHYLPREDNISOLONE THERAPY IN DECEASED DONORS REDUCES INFLAMMATION IN THE DONOR LIVER AND IMPROVES OUTCOME AFTER LIVER TRANSPLANTATION: A PROSPECTIVE RANDOMIZED CONTROLLED TRIAL. ANN SURG 248:1042-1050, 2008
13. SCHNUELLE P, GOTTMANN U, HOEGER S, ET AL.: EFFECTS OF DONOR PRETREATMENT WITH DOPAMINE ON GRAFT FUNCTION AFTER KIDNEY TRANSPLANTATION: A RANDOMIZED CONTROLLED TRIAL. JAMA 302:1067-1075, 2009
14. LASKOWSKI IA, PRATSCHKE J, WILHELM MM, ET AL.: EARLY AND LATE INJURY TO RENAL TRANSPLANTS FROM NON-HEART-BEATING DONORS. TRANSPLANTATION 73:1468-1473, 2002
15. LEMOS FB, IJZERMANS JN, ZONDERVAN PE, ET AL.: DIFFERENTIAL EXPRESSION OF HEME OXYGENASE-1 AND VASCULAR ENDOTHELIAL GROWTH FACTOR IN CADAVERIC AND LIVING DONOR KIDNEYS AFTER ISCHEMIA REPERFUSION. J AM SOC NEPHROL 14:3278-3287, 2003
16. DOLS LF, KOK NF, IJZERMANS JN: SURGICAL ASPECTS OF LIVE KIDNEY DONATION. FRONT BIOSCI 14:4746-4757, 2009
17. SENER A, COOPER M: LIVE DONOR NEPHRECTOMY FOR KIDNEY TRANSPLANTATION. NAT CLIN PRACT UROL 5:203-210, 2008
18. BIRKS EJ, OWEN VJ, BURTON PB, ET AL.: TUMOR NECROSIS FACTOR-ALPHA IS EXPRESSED IN DONOR HEART AND PREDICTS RIGHT VENTRICULAR FAILURE AFTER HUMAN HEART TRANSPLANTATION. CIRCULATION 102:326-331, 2000
19. MURUGAN R, VENKATARAMAN R, WAHED AS, ET AL.: INCREASED PLASMA INTERLEUKIN-6 IN DONORS IS ASSOCIATED WITH LOWER RECIPIENT HOSPITAL-FREE SURVIVAL AFTER CADAVERIC ORGAN TRANSPLANTATION. CRIT CARE MED 36:1810-1816, 2008
20. NIJBOER WN, SCHUURS TA, VAN DER HOEVEN JA, ET AL.: EFFECT OF BRAIN DEATH ON GENE EXPRESSION AND TISSUE ACTIVATION IN HUMAN DONOR KIDNEYS. TRANSPLANTATION 78:978-986, 2004
21. MCKEATING EG, ANDREWS PJ, SIGNORINI DF, ET AL.: TRANSCRANIAL CYTOKINE GRADIENTS IN PATIENTS REQUIRING INTENSIVE CARE AFTER ACUTE BRAIN INJURY. BR J ANAESTH 78:520-523, 1997
22. NIJBOER WN, SCHUURS TA, DAMMAN J, ET AL.: KIDNEY INJURY MOLECULE-1 IS AN EARLY NONINVASIVE INDICATOR FOR DONOR BRAIN DEATH-INDUCED INJURY PRIOR TO KIDNEY TRANSPLANTATION. AM J TRANSPLANT 9:1752-1759, 2009
23. PRATSCHKE J, WILHELM MJ, KUSAKA M, ET AL.: ACTIVATION OF PROINFLAMMATORY GENES IN SOMATIC ORGANS

AS A CONSEQUENCE OF BRAIN DEATH. TRANSPLANT PROC 31:1003-1005, 1999
24. TAKADA M, NADEAU KC, HANCOCK WW, ET AL.: EFFECTS OF EXPLOSIVE BRAIN DEATH ON CYTOKINE ACTIVATION OF PERIPHERAL ORGANS IN THE RAT. TRANSPLANTATION 65:1533-1542, 1998
25. VAN DER HOEVEN JA, PLOEG RJ, POSTEMA F, ET AL.: INDUCTION OF ORGAN DYSFUNCTION AND UP-REGULATION OF INFLAMMATORY MARKERS IN THE LIVER AND KIDNEYS OF HYPOTENSIVE BRAIN DEAD RATS: A MODEL TO STUDY MARGINAL ORGAN DONORS. TRANSPLANTATION 68:1884-1890, 1999
26. LIND MY, HAZEBROEK EJ, BAJEMA IM, ET AL.: EFFECT OF PROLONGED WARM ISCHEMIA AND PNEUMOPERITONEUM ON RENAL FUNCTION IN A RAT SYNGENEIC KIDNEY TRANSPLANTATION MODEL. SURG ENDOSC 20:1113-1118, 2006
27. GOMEZ DEL MORAL M, AVILES B, COLBERGER IK, ET AL.: EXPRESSION OF ADHESION MOLECULES AND RANTES IN KIDNEY TRANSPLANT FROM NONHEART-BEATING DONORS. TRANSPL INT 18:333-340, 2005
28. HAUET T, EUGENE M: A NEW APPROACH IN ORGAN PRESERVATION: POTENTIAL ROLE OF NEW POLYMERS. KIDNEY INT 74:998-1003, 2008
29. METCALFE MS, WHITE SA, SAUNDERS RN, ET AL.: LONG-TERM RESULTS OF RENAL TRANSPLANTATION USING ORGANS FROM NON-HEART-BEATING DONORS. TRANSPLANT PROC 33:826, 2001
30. BROOK NR, WHITE SA, WALLER JR, ET AL.: NON-HEART BEATING DONOR KIDNEYS WITH DELAYED GRAFT FUNCTION HAVE SUPERIOR GRAFT SURVIVAL COMPARED WITH CONVENTIONAL HEART-BEATING DONOR KIDNEYS THAT DEVELOP DELAYED GRAFT FUNCTION. AM J TRANSPLANT 3:614-618, 2003
31. KOTSCH K, FRANCUSKI M, PASCHER A, ET AL.: IMPROVED LONG-TERM GRAFT SURVIVAL AFTER HO-1 INDUCTION IN BRAIN-DEAD DONORS. AM J TRANSPLANT 6:477-486, 2006
32. WAGNER M, CADETG P, RUF R, ET AL.: HEME OXYGENASE-1 ATTENUATES ISCHEMIA/REPERFUSION-INDUCED APOPTOSIS AND IMPROVES SURVIVAL IN RAT RENAL ALLOGRAFTS. KIDNEY INT 63:1564-1573, 2003
33. NATH KA: HEME OXYGENASE-1: A PROVENANCE FOR CYTOPROTECTIVE PATHWAYS IN THE KIDNEY AND OTHER TISSUES. KIDNEY INT 70:432-443, 2006
34. MEGYESI J, ANDRADE L, VIEIRA JM, JR., ET AL.: COORDINATION OF THE CELL CYCLE IS AN IMPORTANT DETERMINANT OF THE SYNDROME OF ACUTE RENAL FAILURE. AM J PHYSIOL RENAL PHYSIOL 283:F810-816, 2002
35. SUSA D, MITCHELL JR, VERWEIJ M, ET AL.: CONGENITAL DNA REPAIR DEFICIENCY RESULTS IN PROTECTION AGAINST RENAL ISCHEMIA REPERFUSION INJURY IN MICE. AGING CELL 8:192-200, 2009
36. WHITE SA, JAIN S, ABSALOM H, ET AL.: INFLUENCE OF DELAYED GRAFT FUNCTION IN RENAL TRANSPLANTS FROM CADAVERIC OR NON-HEART-BEATING DONORS. TRANSPLANT PROC 32:189, 2000
37. PFAFFL MW: A NEW MATHEMATICAL MODEL FOR RELATIVE QUANTIFICATION IN REAL-TIME RT-PCR. NUCLEIC ACIDS RES 29:E45, 2001

Chapter 9

Summary and general discussion

SUMMARY

Organ transplantation is the treatment of choice for end stage organ failure, and one-year results are excellent due to refinements in tissue typing, advancements in organ preservation, surgical techniques and more effective immunosuppressive agents. However, two major problems remain in solid organ transplantation; long-term survival of organ grafts has still not reached its full potential, and the shortage of suitable donor organs is still increasing [1]. Long-term graft survival is significantly hampered by chronic transplant dysfunction (CTD). In the kidney, histologic signs of CTD overlap with the histology of normally ageing kidneys. Clinical observations show an inverse relationship between both donor age and ischemia time and long-term graft survival. The mechanisms by which donor age or ischemia lead to reduced functional lifespan are currently unknown. The work described in this thesis was performed to investigate the contribution of aging mechanisms to ischemia reperfusion injury on the short-, and long-term, and to explore novel therapies to limit organ damage induced by an ischemic insult.

Reactive oxygen species (ROS) produced by normal cellular metabolism and in excess during ischemia and reperfusion after transplantation are a primary source of cellular and DNA damage. Since the introduction of the free radical theory of aging by Harman [2], evidence for a molecular connection between oxidative DNA damage and aging, aging-related disorders and so-called 'premature aging' disorders, or segmental progerias is compelling, which suggests that the mechanisms causing normal aging and long-term organ dysfunction may share a common mechanistic basis. To counteract oxidative DNA damage nature has evolved several efficient, highly conserved DNA repair mechanisms [3]. Nucleotide excision DNA repair (NER) is one such repair system that removes a range of damages [4]. Deficiencies in any of the involved NER proteins give rise to several human inheritable syndromes [5, 6]. These disorders are termed segmental progeriod syndromes, because they exhibit some but not all features of aging, but in an accelerated fashion. The mechanisms by which transplanted organs on the long-term develop chronic transplant dysfunction with features of accelerated aging are largely unknown. Therefore we aimed to answer the question, whether NER, which absence causes segmental progeria, could also be involved in transplant associated ischemia reperfusion injury induced accelerated aging. In chapter 3, we studied whether oxidative stress, induced by renal ischemia reperfusion injury, would lead to increased renal dysfunction on the short-term in NER protein deficient mice. We used mouse models deficient of the NER proteins Cockayne Syndrome A and –B (CS). Contrary to our expectations, we found that these mouse models of CS were less susceptible than wild type animals to surgically induced renal ischemia reperfusion injury. Renal failure-related mortality was significantly reduced in CS mice, kidney function was improved and proliferation was significantly higher in the regenerative phase following ischemic injury. Protection from ischemic damage correlated with a reduced inflammatory response following injury (Chapter 3). Our data suggest that congenital DNA repair deficiency can result in the activation of systemic protective mechanisms resulting in a reduced inflammatory response. The question remained whether oxidative stress would in the long-term lead to functional or histological signs of accelerated renal aging in DNA repair deficient mice. Therefore, in a cohort of mice with different NER deficiencies renal ischemia

reperfusion was induced and functional and histologic data was collected over time. Up to eleven months after ischemia reperfusion injury no difference was found in survival, histology, or kidney function as measured by creatinine, blood urea nitrogen (BUN), albuminuria and glomerular filtration rate (GFR) between NER deficient-, and wild-type mice (Chapter 4). These data are in line with the assumption that unrepaired endogenous DNA lesions may elicit an adaptive response, which protects against acute oxidative injury, with sustained functional outcome in the long-term. Further elucidation of this adaptive response could lead to specific interventions to preserve kidney function on the short- and long-term after ischemic injury.

The adaptive response seen in CS-deficient animals shows functional similarity with the protective effect induced by ischemic preconditioning. Ischemic preconditioning is a procedure that entails brief periods of ischemia that protect against a subsequent longer period of ischemia. In Chapter 5, we sought to determine the role of adenosine and its receptors A_1 and A_3 in protection induced by ischemic preconditioning against cardiac ischemia reperfusion injury as sustained in myocardial infarction or cardiac transplantation. The current consensus is that adenosine is not involved in ischemic preconditioning (IPC) in the rat. We hypothesized that the failure to show a role for adenosine might be due to the use of brief IPC stimuli, and therefore tested the hypothesis that myocardial adenosine receptor activation would play a role in IPC in the rat heart when longer IPC stimuli are employed. In addition, the concept of ischemic preconditioning has been expanded to interorgan protection (i.e. myocardial protection by brief ischemia applied to other organs). We therefore tested the hypothesis that adenosine released by the remote ischemic organ stimulates local afferent nerves, which leads to activation of myocardial adenosine receptors, resulting in protection of the heart. Selective blockade of A_1 (8-SPT) or A_3 receptors (MRS-1191) blunted the protection by classical IPC induced by 15-min cardiac artery occlusion followed by 60 minutes of ischemia and 2 hours of reperfusion. A combination of 10-SPT and MRS-1191 abolished the protective effect of classical IPC, which implies that activation of both A_1 and A_3 receptors contributed to the protection by the 15-minutes cardiac artery occlusion. Inter-organ preconditioning (IOPC) with a 15-minutes mesenteric artery occlusion (MAO15), reduced cardiac infarct size produced by 60 minutes of cardiac artery occlusion. This was abolished by pretreatment with the ganglion blocker hexamethonium, but not when hexamethonium was administered 5 minutes after reperfusion. In contrast, 8-SPT abolished the effect of IOPC when administered either before, or 5 minutes after MAO15, indicating that adenosine receptors downstream of the neurogenic pathway (i.e. in the heart) are involved. Involvement of small intestinal adenosine receptors is suggested by the finding that intramesenteric artery (but not intraportal vein) infusion of adenosine induced a similar level of cardioprotection as MAO15, which was also abolished by hexamethonium (Chapter 5).

Inflammation plays a major role in the pathophysiology of renal ischemic injury [7]. The initial ischemic injury results in up-regulation of adhesion molecules on activated endothelium and release of cytokines, reactive oxygen species (ROS) and eicosanoids. Leukocytes, recruited by chemokines and pro-inflammatory cytokines, potentiate injury by generating more ROS and

eicosanoids, thereby further enhancing inflammation. We have previously shown that synthetic oligopeptides related to the β-chain of the hormone chorionic gonadotropin have anti-inflammatory properties [8-10]. Here, we investigated whether these oligopeptides were able to reduce ischemia reperfusion injury of the kidney. We demonstrated that intravenous administration of oligopeptides around the ischemic period significantly reduced mortality seen after severe renal ischemia reperfusion injury and improved kidney function as measured by serum urea levels. Of the various oligopeptides evaluated, AQGV appeared to be the most potent one. The renoprotective effect of AQGV was associated with decreased renal E-selectin transcripts, decreased neutrophil infiltration, reduced numbers of apoptotic tubular epithelial cells and a reduction of systemic levels of TNF-α, IFN-γ, IL-6 and IL-10. These data suggest that AQGV interferes with the early renal inflammatory response induced by ischemia reperfusion and as such prevents parenchymal damage and organ dysfunction. A dose of 3-10 mg/kg proved to be the most potent with regard to reducing mortality as well as preserving kidney function. Up to 30 mg/kg, no toxicity was observed. AQGV treatment, at either 12 and 24 hours, or at 6 and 12 hours post-reperfusion was not associated with improved survival (Chapter 6).

The cardinal histomorphological hallmark of chronic transplant dysfunction in all parenchymal allografts is transplant vasculopathy. This process is characterized by neo-intima formation due to migration and proliferation of vascular smooth muscle cells into the intima [11]. Apart from their immunosuppressive properties, the immunosuppressive drugs cyclosporin [12, 13], tacrolimus [14], mycophenolate mofetil [15] and methylprednisolone [8, 16] have also been shown to reduce ischemic injury. To investigate whether pre-transplantation treatment of the organ donor (donor pre-treatment) with immunosuppressive agents was able to pharmacologically precondition organs, and reduce transplant vasculopathy, we used the rat aorta transplantation model. We observed significant neointima formation in the untreated control group eight weeks after orthotopic aorta transplantation, as well as in the methylprednisolone, MMF, and rapamycin pre-treated groups. Only tacrolimus exerted a significant inhibitory effect on neointima formation in aorta allografts. In-situ activity of matrix metalloproteinases (MMP) was significantly reduced in grafts treated with rapamycin and tacrolimus compared to controls. Immunohistochemistry revealed a high number of CD4+ cells and high CD4/CD8 ratio only in grafts pre-treated with tacrolimus. Therefore, the mechanism of protection is suggested to be a T cell related reduction in MMP activity (Chapter 7).

Although brain death (BD) is a risk factor for kidney function and survival following kidney transplantation, most donor organs are retrieved from brain dead patients. Improved technology and an increasing gap between supply and demand for donor organs have facilitated an increased use of organs from both living donors (LD) and non heart beating donors (NHB). Superior long-term survival is found in the LD group, whereas organs from brain dead as well as NHB donors show decreased post-transplant function and graft survival [17]. Organs from NHB donors are exposed to prolonged warm ischemia times, and organs from both post-mortem sources are exposed to prolonged cold ischemia times. Studies that have investigated differences in gene expression between these different donors usually use kidney biopsies taken during organ retrieval, after cold

ischemia, or after implantation and subsequent reperfusion. Comparison of these groups is difficult since major differences in the course to organ retrieval exist. Therefore, the aim of our study was to compare the impact of brain dead and asystole to living donation, by obtaining kidney biopsy specimens at the moment of organ retrieval, and at the end of cold ischemia. Furthermore, we assessed the effect of an increasing cold preservation period on gene-expression levels. We found a striking difference between brain death and NHB in renal transcription of inflammatory markers such as IL-1β, IL-6, MCP-1, and E-selectin. High levels of inflammatory cytokines in the donor are associated with decreased graft survival [18, 19]. Inflammation resulted in significant injury of the kidney, as shown by the up-regulation of Kim-1. This gene has been recently identified as a discriminating marker between stable and unstable brain death [20], and as predictor of graft function after BD induced kidney injury [21]. We found a significant up-regulation of HO-1 mRNA in BD donors at the time of explantation. Interestingly, the cyclin-dependant kinase inhibitor p21 was also strongly induced both in NHB and BD donors. Although evidence shows that over-expression of HO-1 can protect kidney transplants against ischemic injury [22, 23], the up-regulation of HO-1 and p21 in our models may be envisioned as indicator of unrepairable damage. Our study further shows that mRNA levels of different genes encountered at explantation remain stable during an 18 hour cold preservation period.
In conclusion, we show a massive up-regulation of inflammatory, injury, and cytoprotective genes in kidney transplants from BD-, but not living-, or NHB donors at the time of graft explantation (Chapter 8). This difference may underlie the observed better graft survival of kidneys from NHB donors [24], and lends further support to the anti-inflammatory treatment of BD donors [25, 26].

FUTURE DIRECTIONS

Ischemia-induced damage becomes apparent during the reperfusion of the transplanted organ. Reactive oxygen species (ROS) are formed, which damage vascular endothelial and other cells by causing oxidative DNA damage and lipid peroxidation in the cell membrane. This is thought to initiate the poorly understood pathogenetic process leading to late graft loss. Oxidative damage induces parenchymal injury and may initially kill a large number of cells, forcing proliferation to replenish the lost cells. Expression of adhesion molecules facilitates infiltration of leukocytes in the kidney, which causes more tissue injury and leads to an innate immune response. Damaged graft cells also facilitate immune recognition via their MHC molecules and thus promote the adaptive immune response (rejection), leading to more tissue injury and cell death. This gives rise to a self-sustaining mechanism with cycles of cell death and proliferation that eventually trigger cellular senescence, exhaustion of the proliferative potential and long-term organ dysfunction [27]. Reducing the initial oxidative hit to donor organs may improve graft function on the short-term and survival of those grafts on the long-term.
Studies in this thesis show that amelioration of ischemic damage is possible through several methods, all characterized by the induction of an adaptive stress response, also known as a hormetic response.

Hormesis is a common biological phenomenon in which exposure to a low intensity stressor induces a general adaptive response that has net beneficial effects on the cellular and/or organismal level, including protection against subsequent, higher-dose exposures as well as to different types of stress [28, 29]. We have shown in chapter 3 and 4 that TC-NER deficiency leads to resistance against acute oxidative stress in renal ischemia reperfusion injury. Our data suggest that specific types of unrepaired endogenous DNA lesions lead to a hormetic response. We refer to this protective effect as "genetic preconditioning".

Ischemic preconditioning is one of the most widely studied types of experimental hormesis. It occurs when an organ (the heart for example) is subjected to a brief mild ischemia. After exposure to a short period of ischemia the cells become resistant to acute oxidative stress induced by a full-blown infarction.

Interestingly, caloric restriction has been proposed to act as a mild stressor that has beneficial effects on health- and lifespan through hormetic mechanisms. Apart from increasing life span, caloric restriction has also been shown to increase the resistance of rodents to acute stress.

The physiology of the protective response observed after caloric restriction shows similarities with the observed increased stress resistance in DNA repair deficient mice. Therefore, we hypothesised that these responses share a common biological basis, and aimed to investigate the effect of caloric restriction in models of acute oxidative damage, and to explore the mechanistic basis of protection in these models.

Data from our laboratory indeed show that periods of caloric restriction or fasting prior to surgery lead to protection against renal ischemia reperfusion injury in mice [30], whereas others have shown that fasting protects mice from the adverse side effects of high dose chemotherapy [31]. Further research will have to be done to elucidate the protective nature of hormetic responses, and the extent to which this response is applicable in medicine. Understanding hormesis and ways to induce a hormetic response could lead to a novel approach in which the body's own protective mechanisms may be tapped to combat ischemia reperfusion injury and other stressors.

REFERENCES

1. LECHLER, R.I., ET AL., ORGAN TRANSPLANTATION--HOW MUCH OF THE PROMISE HAS BEEN REALIZED? NAT MED, 2005. 11(6): P. 605-13.
2. HARMAN, D., AGING: A THEORY BASED ON FREE RADICAL AND RADIATION CHEMISTRY. J GERONTOL, 1956. 11(3): P. 298-300.
3. HOEIJMAKERS, J.H., GENOME MAINTENANCE MECHANISMS FOR PREVENTING CANCER. NATURE, 2001. 411(6835): P. 366-74.
4. BROOKS, P.J., ET AL., THE OXIDATIVE DNA LESION 8,5'-(S)-CYCLO-2'-DEOXYADENOSINE IS REPAIRED BY THE NUCLEOTIDE EXCISION REPAIR PATHWAY AND BLOCKS GENE EXPRESSION IN MAMMALIAN CELLS. J BIOL CHEM, 2000. 275(29): P. 22355-62.
5. BOOTSMA, D., KRAEMER, K. H., CLEAVER, J. & HOEIJMAKERS, J. H. J., IN THE METABOLIC AND MOLECULAR BASIS OF INHERITED DISEASE VOL. 1 (EDS SCRIVER, C. R., BEAUDET, A. L., SLY, W. S. & VALLE, D.) 677-703 (MCGRAW-HILL, NEW YORK, 2001). 2001.
6. LEHMANN, A.R., THE XERODERMA PIGMENTOSUM GROUP D (XPD) GENE: ONE GENE, TWO FUNCTIONS, THREE DISEASES. GENES DEV, 2001. 15(1): P. 15-23.
7. BONVENTRE, J.V. AND J.M. WEINBERG, RECENT ADVANCES IN THE PATHOPHYSIOLOGY OF ISCHEMIC ACUTE RENAL FAILURE. J AM SOC NEPHROL, 2003. 14(8): P. 2199-210.
8. AYDIN, G., S.E. OKIYE, AND H. ZINCKE, A COMPARATIVE STUDY OF SEVERAL AGENTS ALONE AND COMBINED IN PROTECTION OF THE RODENT KIDNEY FROM WARM ISCHAEMIA: METHYLPREDNISOLONE, PROPRANOLOL, FUROSEMIDE, MANNITOL, AND ADENOSINE TRIPHOSPHATE-MAGNESIUM CHLORIDE. UROL RES, 1983. 11(3): P. 105-9.
9. KHAN, N.A., ET AL., INHIBITION OF DIABETES IN NOD MICE BY HUMAN PREGNANCY FACTOR. HUM IMMUNOL, 2001. 62(12): P. 1315-23.
10. KHAN, N.A., ET AL., INHIBITION OF SEPTIC SHOCK IN MICE BY AN OLIGOPEPTIDE FROM THE BETA-CHAIN OF HUMAN CHORIONIC GONADOTROPHIN HORMONE. HUM IMMUNOL, 2002. 63(1): P. 1-7.
11. MITCHELL, R.N. AND P. LIBBY, VASCULAR REMODELING IN TRANSPLANT VASCULOPATHY. CIRC RES, 2007. 100(7): P. 967-78.
12. BES, S., ET AL., DIRECT, PLEIOTROPIC PROTECTIVE EFFECT OF CYCLOSPORIN A AGAINST SIMULATED ISCHEMIA-INDUCED INJURY IN ISOLATED CARDIOMYOCYTES. EUR J PHARMACOL, 2005. 511(2-3): P. 109-20.
13. SINGH, D., V. CHANDER, AND K. CHOPRA, CYCLOSPORINE PROTECTS AGAINST ISCHEMIA/REPERFUSION INJURY IN RAT KIDNEYS. TOXICOLOGY, 2005. 207(3): P. 339-47.
14. SAKR, M., ET AL., THE PROTECTIVE EFFECT OF FK506 PRETREATMENT AGAINST RENAL ISCHEMIA/REPERFUSION INJURY IN RATS. TRANSPLANTATION, 1992. 53(5): P. 987-91.
15. VENTURA, C.G., ET AL., MYCOPHENOLATE MOFETIL ATTENUATES RENAL ISCHEMIA/REPERFUSION INJURY. J AM SOC NEPHROL, 2002. 13(10): P. 2524-33.
16. GLANEMANN, M., ET AL., ISCHEMIC PRECONDITIONING AND METHYLPREDNISOLONE BOTH EQUALLY REDUCE HEPATIC ISCHEMIA/REPERFUSION INJURY. SURGERY, 2004. 135(2): P. 203-14.
17. KANEKU, H.K. AND P.I. TERASAKI, THIRTY YEAR TREND IN KIDNEY TRANSPLANTS: UCLA AND UNOS RENAL TRANSPLANT REGISTRY. CLIN TRANSPL, 2006: P. 1-27.
18. MURUGAN, R., ET AL., INCREASED PLASMA INTERLEUKIN-6 IN DONORS IS ASSOCIATED WITH LOWER RECIPIENT HOSPITAL-FREE SURVIVAL AFTER CADAVERIC ORGAN TRANSPLANTATION. CRIT CARE MED, 2008. 36(6): P. 1810-6.
19. NIJBOER, W.N., ET AL., EFFECT OF BRAIN DEATH ON GENE EXPRESSION AND TISSUE ACTIVATION IN HUMAN DONOR KIDNEYS. TRANSPLANTATION, 2004. 78(7): P. 978-86.
20. SCHUURS, T.A., ET AL., DISTINCT TRANSCRIPTIONAL CHANGES IN DONOR KIDNEYS UPON BRAIN DEATH INDUCTION IN RATS: INSIGHTS IN THE PROCESSES OF BRAIN DEATH. AM J TRANSPLANT, 2004. 4(12): P. 1972-81.
21. NIJBOER, W.N., ET AL., KIDNEY INJURY MOLECULE-1 IS AN EARLY NONINVASIVE INDICATOR FOR DONOR BRAIN DEATH-INDUCED INJURY PRIOR TO KIDNEY TRANSPLANTATION. AM J TRANSPLANT, 2009. 9(8): P. 1752-9.
22. KOTSCH, K., ET AL., IMPROVED LONG-TERM GRAFT SURVIVAL AFTER HO-1 INDUCTION IN BRAIN-DEAD DONORS. AM J TRANSPLANT, 2006. 6(3): P. 477-86.
23. NATH, K.A., HEME OXYGENASE-1: A PROVENANCE FOR CYTOPROTECTIVE PATHWAYS IN THE KIDNEY AND OTHER TISSUES. KIDNEY INT, 2006. 70(3): P. 432-43.
24. WHITE, S.A., ET AL., INFLUENCE OF DELAYED GRAFT FUNCTION IN RENAL TRANSPLANTS FROM CADAVERIC OR NON-HEART-BEATING DONORS. TRANSPLANT PROC, 2000. 32(1): P. 189.

25. KOTSCH, K., ET AL., METHYLPREDNISOLONE THERAPY IN DECEASED DONORS REDUCES INFLAMMATION IN THE DONOR LIVER AND IMPROVES OUTCOME AFTER LIVER TRANSPLANTATION: A PROSPECTIVE RANDOMIZED CONTROLLED TRIAL. ANN SURG, 2008. 248(6): P. 1042-50.
26. SCHNUELLE, P., ET AL., EFFECTS OF DONOR PRETREATMENT WITH DOPAMINE ON GRAFT FUNCTION AFTER KIDNEY TRANSPLANTATION: A RANDOMIZED CONTROLLED TRIAL. JAMA, 2009. 302(10): P. 1067-75.
27. NANKIVELL, B.J. AND J.R. CHAPMAN, CHRONIC ALLOGRAFT NEPHROPATHY: CURRENT CONCEPTS AND FUTURE DIRECTIONS. TRANSPLANTATION, 2006. 81(5): P. 643-54.
28. MATTSON, M.P., HORMESIS DEFINED. AGEING RES REV, 2008. 7(1): P. 1-7.
29. MURRY, C.E., R.B. JENNINGS, AND K.A. REIMER, PRECONDITIONING WITH ISCHEMIA: A DELAY OF LETHAL CELL INJURY IN ISCHEMIC MYOCARDIUM. CIRCULATION, 1986. 74(5): P. 1124-36.
30. MITCHELL, J.R., ET AL., SHORT-TERM DIETARY RESTRICTION AND FASTING PRECONDITION AGAINST ISCHEMIA REPERFUSION INJURY IN MICE. AGING CELL. 9(1): P. 40-53.
31. RAFFAGHELLO, L., ET AL., STARVATION-DEPENDENT DIFFERENTIAL STRESS RESISTANCE PROTECTS NORMAL BUT NOT CANCER CELLS AGAINST HIGH-DOSE CHEMOTHERAPY. PROC NATL ACAD SCI U S A, 2008. 105(24): P. 8215-20.

Nederlandse samenvatting

Orgaan transplantatie is de behandeling van keuze voor eind stadium orgaanfalen. De 1-jaars resultaten zijn tegenwoordig uitstekend door verfijning van weefsel typering, vorderingen in preservatie van organen, chirurgische technieken en effectievere immunosuppresiva. Echter, twee complexe problemen blijven bestaan in het veld van orgaan transplantatie; lange-termijn overleving van getransplanteerde organen heeft nog steeds zijn volle potentie niet bereikt en het gebrek aan geschikte organen neemt steeds verder toe. Lange-termijn overleving van organen wordt significant beperkt door chronische transplantaat disfunctie (CTD). In de nier overlappen de histologische kenmerken van CTD met die van normaal ouder wordende nieren. Klinische observatie laat een omgekeerd evenredige correlatie zien tussen donor leeftijd en ischemietijd / preservatietijd en lange termijn transplantaat overleving. Waardoor hogere donorleeftijd of ischemietijd leiden tot een verkorte functionele overleving van getransplanteerde organen is tot op heden niet bekend.

Het werk beschreven in dit proefschrift is verricht om enerzijds te onderzoeken wat de bijdrage van verouderingsmechanismen is in ischemie reperfusie schade op de korte en lange termijn en om anderzijds nieuwe therapieën te ontwikkelen om orgaanschade na een ischemisch insult te beperken.

Zuurstof radicalen, geproduceerd tijdens normaal cellulair metabolisme en in overmaat tijdens ischemie en reperfusie, zijn een primaire bron voor cellulaire en DNA schade. Sinds de introductie van de vrije radicalen theorie van Harman in 1956, is er steeds meer bewijs voor een moleculaire connectie tussen oxidatieve DNA schade, veroudering, verouderingsgerelateerde ziekte en de premature verouderingssyndromen, ook wel segmentale progeria genoemd. Dit suggereert dat de mechanismen die leiden tot normale veroudering en tot lange termijn orgaandisfunctie mogelijk een zelfde mechanistische basis delen. Om oxidatieve DNA schade tegen te gaan, zijn er een aantal efficiënte en geconserveerde DNA reparatiemechanismen geëvolueerd. Nucleotide excisie DNA reparatie (NER) is een van deze mechanismen. Deficiënties in een van de NER eiwitten kan aanleiding geven tot een aantal erfelijke humane syndromen. Deze afwijkingen worden segmentale progeria syndromen genoemd, aangezien zij een deel van de kenmerken van veroudering in een versneld tempo laten zien. De mechanismen waardoor getransplanteerde organen op de lange termijn chronische transplantaat disfunctie met versnelde verouderingskenmerken ontwikkelen, zijn grotendeels onbekend. Ons eerste doel was om de vraag te beantwoorden of NER betrokken is bij door ischemie reperfusie schade geïnduceerde versnelde veroudering. In hoofdstuk 3 bestudeerden wij of oxidatieve stress geïnduceerd door renale ischemie reperfusie schade, zou leiden tot toename van renale disfunctie op korte termijn in NER eiwit deficiënte muizen. Daarvoor gebruikten wij een muismodel met een deficiëntie van de NER eiwitten Cockayne Syndrome A en –B (CS). In tegenstelling tot onze verwachting vonden we dat deze muismodellen van CS minder vatbaar waren dan wild-type muizen voor chirurgisch geïnduceerde

renale ischemie reperfusie schade. Nierfalen gerelateerde mortaliteit was significant minder in CS muizen, nierfunctie was verbeterd en proliferatie was significant hoger in de regeneratieve fase na het ischemisch insult. Bescherming tegen ischemische schade correleerde met een verminderde inflammatoire respons na het insult (Hoofdstuk 3). Onze data suggereren dat congenitale DNA reparatie deficiëntie kan leiden tot een activatie van systemische protectieve mechanismen, resulterend in een verminderde inflammatoire respons. De vraag blijft echter of oxidatieve stress op de lange termijn leidt tot functionele of histologische kenmerken van versnelde veroudering in DNA reparatie deficiënte muizen. Om dit te bestuderen werd een cohort van verschillende DNA reparatie deficiënte muizen onderworpen aan renale ischemie reperfusie schade en werden functionele en histologische gegevens verzameld. Tot 11 maanden na ischemie reperfusie schade werd geen verschil gevonden tussen NER deficiënte-, en wild-type muizen in overleving, histologie en nierfunctie (Hoofdstuk 4). Deze gegevens corresponderen met de aanname dat ongerepareerde endogene DNA lesies zouden kunnen leiden tot een adaptieve respons, met als resultaat bescherming tegen acute oxidatieve schade met behoud van nierfunctie op lange termijn. Verdere dissectie van deze adaptieve respons kan leiden tot specifieke interventies om de nierfunctie te behouden op korte en lange termijn na een ischemisch insult.

De adaptieve respons in CS deficiënte dieren heeft functionele overeenkomsten met het beschermende effect dat wordt gezien bij ischemische preconditionering. Ischemische preconditionering is een procedure waarbij perioden van korte ischemie bescherming bieden tegen een daaropvolgende langere periode van ischemie. In hoofdstuk 5 hebben we de rol van adenosine en zijn receptoren A_1 en A_3 bestudeerd in de bescherming, geïnduceerd door ischemische preconditionering, tegen cardiale ischemie reperfusie schade, zoals gezien wordt bij een hartinfact of harttransplantatie. De consensus was dat adenosine niet betrokken is bij ischemische preconditionering (IPC) in de rat. Wij hypothetiseerden dat het niet aantonen van een rol voor adenosine lag in een te korte ischemische prikkel. Daarvoor onderzochten wij de hypothese dat myocardiale adenosine receptor activatie wel aangetoond kon worden, wanneer langere IPC prikkels gegeven werden. Daarnaast is het concept van ischemische preconditionering doorgetrokken naar het concept van inter-orgaan bescherming (myocardiale bescherming door kortdurende ischemie aan andere organen). Om dit te onderzoeken werd de hypothese getest dat adenosine, vrijgekomen in een ander orgaan, de lokaal afferente zenuwen stimuleert. Dit zorgt voor activatie van myocardiale adenosine receptoren, leidend tot bescherming van het hart. Selectieve blokkade van de A_1 (8-SPT) of A_3 receptoren (MRS-1191) verminderde het beschermingseffect door ischemische preconditionering door 15 minuten cardiaal arterie occlusie (CAO15) gevolgd door 60 minuten ischemie en 2 uur reperfusie. De combinatie van 8-SPT en MRS-1191 zorgde voor volledige opheffing van het beschermende effect van ischemische preconditionering. Daaruit kan de conclusie getrokken worden dat activatie van beide A_1 en A_3 receptoren bijdraagt aan het beschermende effect door CAO15. Inter-orgaan preconditionering (IOPC) door 15 minuten mesenteriaal arterie occlusie (MAO15), reduceerde het cardiale infarct gebied na 60 minuten cardiaal arterie occlusie. Dit effect kon worden opgeheven door toediening van de ganglion blocker hexamethonium voor MAO15. Echter, bij toediening van hexamethonium na MAO15 bleef het

beschermende effect gehandhaafd. In tegenstelling hiermee kon toediening van 8-SPT voor of na MAO15 het effect van IOPC opheffen, een indicatie dat adenosine receptoren voorbij de zenuwbanen (het hart) betrokken zijn. Betrokkenheid van adenosine receptoren in de dunne darm is suggestief, aangezien infusie van adenosine in de mesenteriaal arterie (maar niet in de portale vene) dezelfde cardiale bescherming geeft als MAO15 (Hoofdstuk 5).

Inflammatie speelt een belangrijke rol in de pathofysiologie van renale ischemie reperfusie schade. De initiële ischemische schade zorgt voor op-regulatie van adhesie moleculen op het geactiveerde endotheel en het vrijkomen van cytokinen en zuurstof radicalen. Leukocyten, gerecruteerd door chemokinen en pro-inflammatoire cytokinen versterken de schade door vorming van meer zuurstof radicalen en cytokinen, het inflammatoire proces daardoor versterkend. Wij hebben eerder laten zien dat synthetische oligopeptiden gerelateerd aan de β-keten van het hormoon chorion gonadotropine anti-inflammatoire kenmerken bezitten. Hier hebben wij onderzocht of deze oligopeptiden ischemie reperfusie schade aan de nier kunnen beperken. Wij lieten zien dat intraveneuze toediening van oligopeptiden rond de ischemische periode een significante daling van mortaliteit laat zien na ischemie reperfusie schade aan de nier en een verbeterde nierfunctie bewerkstelligt. Van de onderzochte oligopeptiden komt AQGV als meest potent naar voren. Het beschermende effect was geassocieerd met een verlaging van E-selectine transcriptie, verlaging van infiltratie van neutrofielen, vermindering van het aantal apoptotische tubulaire epitheelcellen en een verlaging van systemische spiegels van TNF-α, IFN-γ, IL-6 en IL-10. Deze data suggereren dat AQGV interfereert met de vroege renale inflammatoire respons geïnduceerd door ischemie reperfusie en daardoor parenchymateuze schade en orgaandisfunctie voorkomt. Een dosis tussen 3 – 10 mg/kg bleek het meest potent in verlaging van de mortaliteit en behoud van nierfunctie. Tot 30 mg/kg werd geen toxiciteit aangetoond. AQGV toediening op 12 en 24 uur, of op 6 en 12 uur post-reperfusie had geen effect op de overleving vergeleken met controles (Hoofdstuk 6).

Het belangrijkste histomorfologische kenmerk van chronische transplantaat disfunctie in alle getransplanteerde organen is transplantaat vasculopathie. Dit proces wordt gekenmerkt door neo-intima formatie door migratie en proliferatie van vasculaire gladde spiercellen in de intima. Immunosuppressiva zoals cyclosporine, tacrolimus, mycophenolate mofetil (MMF) en methylprednison hebben naast hun bekende immunosuppressieve effecten ook laten zien dat zij ischemie reperfusie schade kunnen beperken. Om te onderzoeken of pre-transplantatie behandeling van de orgaandonor met immunosuppressiva een farmacologisch preconditioneringseffect kon bewerkstelligen en daardoor transplantaat vasculopathie kon remmen, hebben wij een aorta transplantatie model in de rat gebruikt. Wij vonden een significante neointima formatie in de onbehandelde groep 8 weken na een orthotopische aorta transplantatie, net als in de met methylprednison, MMF en rapamycine behandelde groepen. Alleen tacrolimus liet een significant remmend effect zien op neointima formatie in aortatransplantaten. De in-situ activiteit van matrix metalloproteinases (MMP) was significant verminderd in aortatransplantaten behandeld met rapamycine en tacrolimus vergeleken met de controle groep. Immunohistochemische kleuring liet een hoog aantal CD4+ cellen en een hoge CD4/CD8 ratio zien in tacrolimus behandelde

transplantaten. Daarmee lijkt het mechanisme van bescherming een T-cel gerelateerde reductie van MMP activiteit te zijn (Hoofdstuk 7).

Alhoewel hersendood (HD) een risico factor is voor nierfunctie en transplantaatoverleving na niertransplantatie, zijn de meeste donororganen afkomstig van hersendode patiënten. Verbetering in technologie en de toegenomen vraag naar donororganen heeft gezorgd dat er meer gebruik wordt gemaakt van organen van levende donoren (LD) en non heart beating (NHB) donoren. Superieure lange termijn overleving wordt gevonden in de groep van de levende donoren, terwijl de organen van HD en NHB donoren een verminderde post-transplantatie functie en overleving laten zien. Organen van NHB donoren zijn blootgesteld aan verlengde warme ischemietijden en tevens zijn organen van beide post-mortem groepen blootgesteld aan lange koude ischemietijden. Onderzoeken die de verschillen in genexpressie tussen verschillende donorgroepen hebben onderzocht, gebruiken nierbiopten afgenomen gedurende explantatie, na koude ischemie of na implantatie en daaropvolgende reperfusie. Vergelijking van deze groepen is lastig aangezien er grote verschillen bestaan in de afname momenten. Derhalve was het doel van dit onderzoek om de rol van hersendood en asystole in vergelijking tot levende donatie te onderzoeken door afname van nierbiopten op het moment van orgaan uitname en aan het eind van de koude ischemie. Verder onderzochten wij wat het effect was van de duur van de koude ischemietijd op genexpressie. Wij vonden een groot verschil in expressie van ontstekingsgenen (IL-1β, IL-6, MCP-1 en E-selectine) tussen HD en NHB groep. De ontstekingsreactie zorgde voor significante schade aan de nier, aangetoond door de verhoogde expressie van Kim-1. Er was een verhoogde expressie van HO-1 in HD op het moment van uitname. Het gen p21 was ook sterk geïnduceerd in zowel HD en NHB donoren. Alhoewel er bewijs bestaat dat overexpressie van HO-1 het niertransplantaat kan beschermen tegen ischemische schade, lijkt de overexpressie van HO-1 en p21 in ons model meer een uiting te zijn van schade. Verder laat dit onderzoek zien dat er geen verandering is in genexpressie tussen het moment van orgaan uitname tot 18 uur koude ischemie (Hoofdstuk 8).

Dankwoord

Na al die jaren is het eindelijk zo ver. Het proefschrift is klaar. Veel mensen hebben op enige wijze bijgedragen aan de totstandkoming van dit proefschrift. In het bijzonder zou ik de volgende personen willen bedanken.

Mijn copromotor dr. R.W.F. de Bruin. Ron, vanaf het begin heb jij met jouw ontspannen, doch gedreven begeleiding een stevige basis gecreëerd voor dit proefschrift. Ondanks dat er momenten waren dat ik door de bomen het bos niet meer zag, kon jij mij op de juiste manier stimuleren gericht te kijken om het proefschrift te voltooien. Daarnaast wil ik je zeer bedanken voor de waardevolle vriendschap die is ontstaan onderweg naar het viaduct in Boston.

Mijn promotor prof.dr. J.N.M IJzermans. Jan, dank voor de gelegenheid die je mij hebt geboden tot het verrichten van dit onderzoek en de mogelijkheid om naast het onderzoek ook transplantatiecoördinator te zijn. Jouw kritische blik op het geheel en nuttige suggesties zijn voor mij zeer waardevol geweest.

Dr. J.R. Mitchell. Jay, thank you for your enormous contribution to our collaboration. Even when our results were the opposite of our hypothesis, you could always keep up the spirit and create a new hypothesis to further explore. Good luck with your new lab in Boston!

Prof.dr. D.J.G.M. Duncker. Dirk, dank voor de mogelijkheid die jij en prof.dr. P.D. Verdouw mij hebben geboden om onderzoek te verrichten op de Experimentele Cardiologie.

De overige leden van de promotiecommissie: prof.dr. J.H.J. Hoeijmakers en prof.dr. R. Benner, dank voor de prettige samenwerking, het beoordelen van het manuscript en de waardevolle suggesties.

Alle medewerkers en collegae in het Laboratorium voor Experimentele Chirurgie voor de prettige samenwerking door de jaren heen. Sandra van den Engel voor de gigantische hulp met het uitvoeren van de verschillende experimenten en kleuringen. Dr. H.P. Roest, Henk dank voor de waardevolle opzet van de moleculair biologische technieken. Pim van Schalkwijk voor de verwerking van de honderden plasma- en urinemonsters. Fred Bonthuis, Martin Huisman en Kristin Mangundap, voor jullie hulp bij de experimenten.

Dr. I.M. Bajema. Ingeborg, dank voor het beoordelen van alle coupes.

Dr. R.M.H. Roumen en dr. M.R.M. Scheltinga. Rudi, Marc, opleider en plaatsvervangend opleider, en overige maatschapsleden, dank voor de ruimte die jullie mij tijdens de jaren in Veldhoven hebben gegeven om naast de opleiding mijn onderzoek af te ronden. Daarnaast dank voor de onvergetelijke en leerzame tijd!

Manon Hofstra, Anneloes van den Berg en Mentha Hoekstra @ Dare to Design, dank voor het vormgeven en inkleuren van dit proefschrift.

Mijn paranimfen, Armand van Kanten en Maarten Loos. Armand, vrienden sinds mijn co-schap Heelkunde in het Zuiderziekenhuis. Hopelijk volgen er meer OK-dagen in het AZP. Maarten, mede skireis-organisator. Wij zien elkaar weer in "The Future". Beiden dank voor jullie ondersteuning op 3 november.

Mijn ouders, lieve mama en papa. Dank voor alle mogelijkheden die jullie hebben gecreëerd voor Danijela en mij. Voor jullie onvoorwaardelijke liefde en steun! Betere ouders zijn er niet.

Mijn zus Danijela, veel succes met het afronden van jouw opleiding tot kinderarts "down under" en veel liefde toegewenst aan jou, Max, Eva en David.

Allerliefste Bianca, jij bent mijn alles.
Allerliefste Lucille, wat een mooi stralend lichtje ben jij.
Wat is het leven mooi met jullie.

Curriculum Vitae

Denis Susa werd geboren op 27 april 1975 te Rotterdam. Het Voorbereidend Wetenschappelijk Onderwijs werd genoten op Het Rotterdamsch Lyceum te Rotterdam. Door uitloting werd van 1993 tot 1995 de studie Farmacie gevolgd aan de Universiteit van Utrecht. In 1994 begon hij met de studie Geneeskunde aan de Erasmus Universiteit te Rotterdam. Tijdens de studie werd de propedeuse Farmacie behaald; het afstudeeronderzoek werd verricht op de afdeling Experimentele Cardiologie bij prof. dr. P.D. Verdouw en prof. dr. D.J. Duncker. In 2002 behaalde hij het artsexamen, waarna hij een aanstelling kreeg als arts-onderzoeker op het Laboratorium voor Experimentele Chirurgie van het Eramus Medisch Centrum, onder begeleiding van prof. dr. J.N.M. IJzermans en dr. R.W.F. de Bruin. In 2004 was hij naast arts-onderzoeker tevens transplantatie-coördinator voor West-Nederland. In 2005 werkte hij als AGNIO chirurgie in het Amphia Ziekenhuis te Breda. Op 1 januari 2006 is hij begonnen met de opleiding tot chirurg in het Maxima Medisch Centrum te Veldhoven (dr. R.M.H. Roumen). Na een stage van 3 maanden in het St. Elisabeth Hospitaal te Curaçao (dr. P.R. Fa-Si-Oen), is hij per 1 januari 2010 begonnen met het academisch gedeelte in het Universitair Medisch Centrum St. Radboud te Nijmegen (prof. dr. C.J.H.M van Laarhoven) als differentiant chirurgische oncologie. Per 1 januari 2011 zal hij in zijn laatste jaar de differentiatie longchirurgie doen in het Canisius Wilhelmina Ziekenhuis te Nijmegen (dr. W.B. Barendregt).

PhD Portfolio

Summary of PhD training and teaching

Name PhD student: Denis Susa Erasmus MC Department: Surgery Research School: Molecular Medicine	PhD period: 2002 - 2010 Promotor: prof.dr. J.N.M. IJzermans Supervisor: dr. R.W.F. de Bruin	
1. PhD training		
	Year	**Workload Hours/ ECTS**
General courses		
- Laboratory Animal Science	1998	
- Evidence Based Medicine	2007	1 ECT
Specific courses (e.g. Research school, Medical Training)		
- ANIOS Surgery	2005	
- AIOS Surgery	2006 - 2010	
Seminars and workshops		
- Basis course Microsurgery, Erasmus MC, Rotterdam	2002	1 ECT
- Basic Immunology, ATC, Washington DC, USA	2003	1 ECT
- Communication training, Transcriptum, Rotterdam	2004	1 ECT
- Surgical Aspects of Organ Procurement, LUMC, Leiden	2004	1 ECT
- Auteurscursus, NTvG, Amsterdam	2006	1 ECT
Presentations		
- **National Conference**		
- Wenckebach Institute, Groningen	2002	1 ECT
- SEOHS, Rotterdam	2002	1 ECT
- Stafdag Heelkunde, Rotterdam	2003	1 ECT
- Congres Nederlandse Transplantatie Vereniging, Rockanje	2003	1 ECT
- Stafdag Heelkunde, Rotterdam	2003	1 ECT
- Congres Nederlandse Transplantatie Vereniging, Maastricht	2005	1 ECT
- **International Conference**		
- Cold Spring Harbor Laboratory Meeting, Molecular Genetics of Aging New York, USA	2002	1 ECT
- ESSR, Gent, Belgium	2003	1 ECT
- American Transplantation Congress, Seattle, USA	2005	1 ECT
- ESOT, Prague, Tsjechia	2007	2 ECT
(Inter)national conferences		
- Congres Nederlandse Transplantatie Vereniging , Eden, The Netherlands	2002	1 ECT
- American Transplantation Congress, Washington DC, USA	2003	1 ECT
- American Transplantation Congress, Boston, USA	2004	1 ECT
2. Teaching		
	Year	**Workload Hours/ ECTS**
Supervising		
- Supervising student graduation projects	2002 - 2004	5 ECT