R O T R F

Roche Organ Transplantation Research Foundation

BIANNUAL REPORT

April 2002

The Roche Organ Transplantation Research Foundation

The mission of the Roche Organ Transplantation Research Foundation (ROTRF) is to advance the science of solid organ transplantation in order to improve the care of the thousands of patients undergoing transplantation every year. The results of the funded research projects will contribute to an understanding of many aspects of the clinical and scientific adventure of transplantation, e.g. the mechanisms of long-term organ deterioration, the consequences of tissue injury, and the opportunities to intervene in these processes.

The Foundation is an independent medical research charity that provides operating funds to established academic staff in universities, transplant centres and research institutes. The Foundation supports research in solid organ transplantation, particularly where there is an unmet medical need.

The funding of the Foundation consists of a donation from F. Hoffmann-La Roche Ltd, with an initial sum of 25 million Swiss francs over the first five years and a renewal donation of 15 million Swiss francs over the following three years. The funds are disbursed as grants of up to 300,000 Swiss francs distributed over three years. The Foundation is legally independent from Roche and is guided solely by the Board of Trustees according to its charter.

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1. Preface

On behalf of the Board of Trustees, I am pleased to announce that eleven research grants have been awarded to scientists around the world following the seventh cycle of grant review. A total of 2.1 million Swiss Francs (CHF) has been awarded in this funding cycle.

The Progress Reports published in this and previous Biannual Reports demonstrate the quality of work that has been carried out on ROTRF grants. Many papers have already been published in respected international journals, and it is to be expected that the number of papers will grow considerably over the next few years. Thus, the ROTRF is fulfilling its mission to advance the science of solid organ transplantation.

The standard of research that has already been carried out with ROTRF funds was also demonstrated in a satellite symposium entitled "Highlights of Projects Supported by the Roche Organ Transplantation Research Foundation (ROTRF)" that took place at the Fifth International Congress on New Trends in Immunosuppression in Geneva in February of this year. The symposium was sponsored by the ROTRF and F. Hoffmann-La Roche Ltd and was the first symposium that dealt solely with research projects funded by the ROTRF.

The symposium was chaired by Prof. Pekka Häyry and myself. Eight international scientists presented the results of their work that has been supported by the ROTRF. The data presented highlighted the originality and diversity of work supported by the ROTRF and demonstrated to an international audience the beneficial role that the ROTRF plays in transplantation research worldwide. We were particularly pleased that many of the presenters, and indeed the recipients of ROTRF funds, are young investigators, and that the ROTRF funds were important not only in supporting their projects but also in establishing their investigative careers.

In summary, the Foundation is a great and unique achievement, and all involved can be proud of it. All those who have been involved in supporting the ROTRF to accomplish its mission, especially Roche by funding this independent Foundation, deserve the Foundation's gratitude.

On behalf of the Board of Trustees

Phil Halloran

2. Facts and Figures

Funding Cycle VII – Letter of Intent Submission in October 2001

In the seventh ROTRF funding cycle, 124 letters of intent were received from scientists around the world. Over half of the applications came from the United States (54%), while 31% came from Europe, the major country being the UK (17%). Asia, Oceania, South America and Canada accounted for the remaining 15% of the applicants.

The Scientific Advisory Committee of the ROTRF evaluated all the applications, which were submitted electronically via the ROTRF homepage (www.ROTRF.org), on the basis of originality and scientific excellence. The top 22 applications were invited to submit full paper applications, and the 21 applications received subsequently underwent a second thorough review by the Scientific Advisory Committee and the Board of Trustees.

ROTRF Grants were finally awarded to 11 applicants: seven from the USA; and one each from Australia, Belgium, France and Italy (see the pink dots on the world map on the following page).

The abstracts of the novel and promising research projects that received funding in cycle VII are presented on the following pages. Their research interests focus mainly on the improvement of long-term graft survival and prevention of chronic organ dysfunction, induction of tolerance, the development of new immunosuppressive agents, and the improvement of donor organ preservation.

In this seventh cycle of ROTRF Grant Awards, a total of 2.1 million Swiss francs was allocated.

Statistics on Applications to the ROTRF

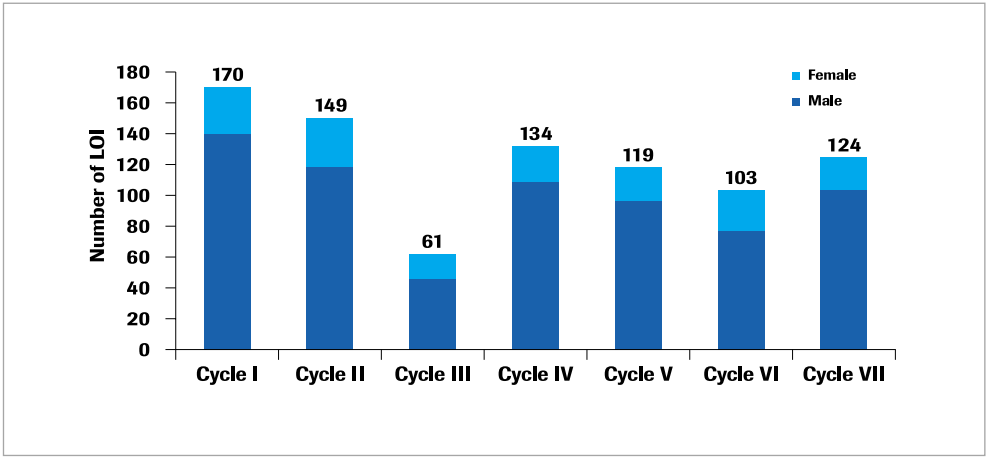


Figure 1. *Number and gender of applicants who submitted letters of intent (LOI) to the ROTRF during the first seven ROTRF funding cycles.*

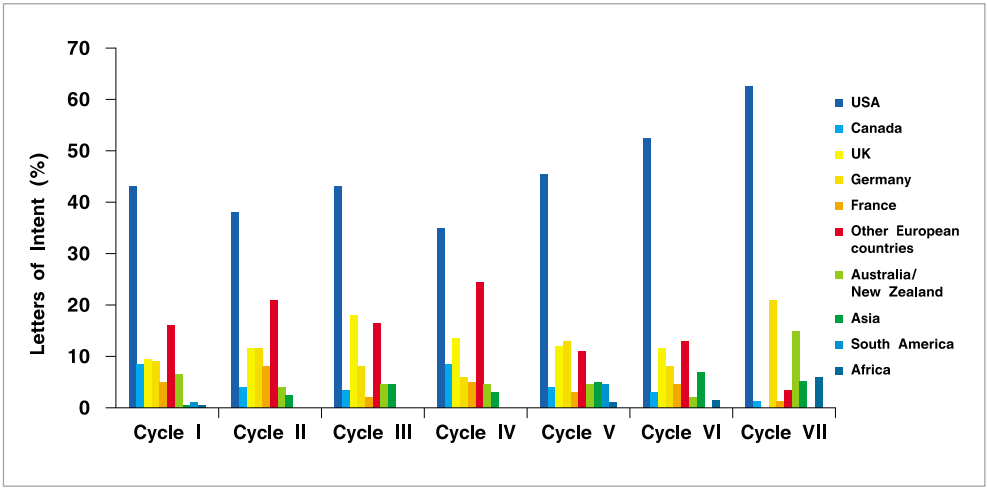
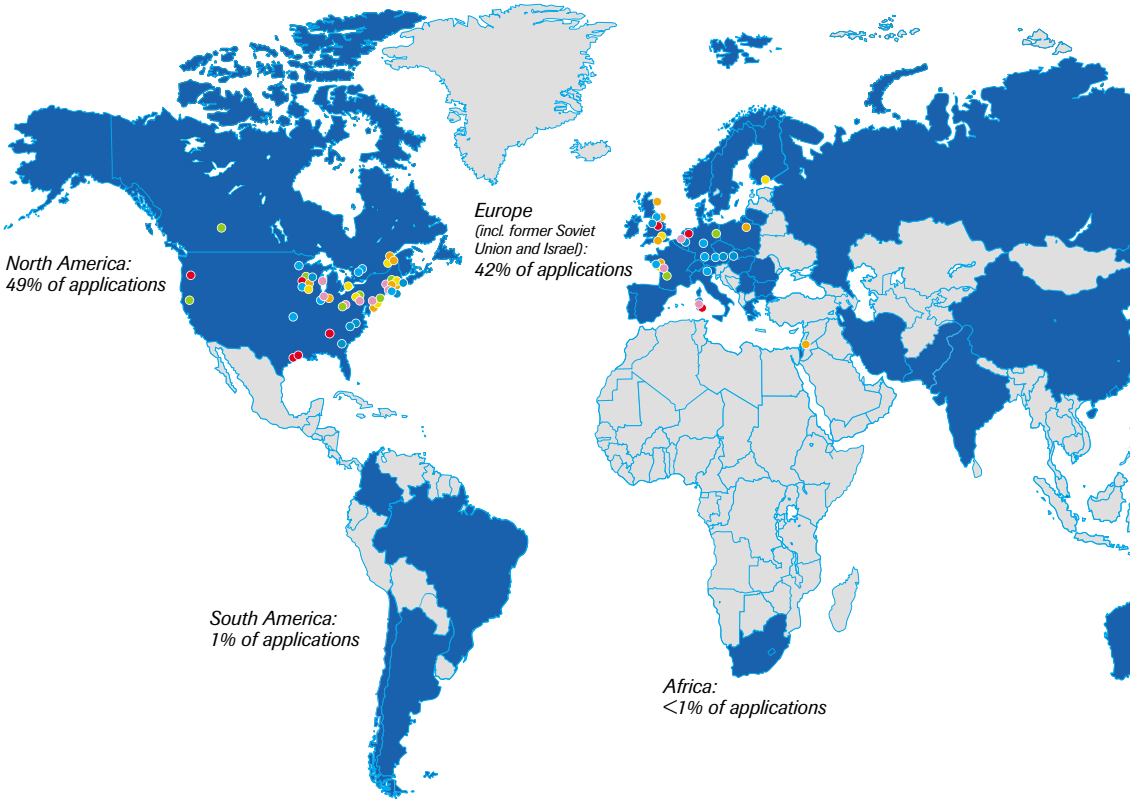


Figure 2. *Geographical distribution of the applicants who submitted letters of intent (LOI) during the first seven ROTRF funding cycles.*

The Global View of Applications to the ROTRF

Distribution of ROTRF applications worldwide



Asia and The Middle East: 3% of applications

Australia: 5% of applications

at least one application ever received

no application received

Cycle I Grantees

Berlin, Germany
Bordeaux, France
Boston, USA
Cincinnati, USA
Edmonton, Canada
Madison, USA
Melbourne, Australia
New Haven, USA
Pittsburgh, USA
San Francisco, USA

Cycle II Grantees

Boston, USA
Helsinki, Finland
London, Canada
Madison, USA
Montreal, Canada
Nantes, France
New York, USA
Oxford, UK
Pittsburgh, USA

Cycle III Grantees

Atlanta, USA
Birmingham, UK
Cagliari, Italy
Houston, USA
Houston, USA
Madison, USA
Nijmegen, The Netherlands
Portland, USA
Winnipeg, Canada

Cycle IV Grantees

Boston, USA
Boston, USA
Chicago, USA
Dundee, UK
Laval, Canada
Madison, USA
Montreal, Canada
Nantes, France
Newcastle-upon-Tyne, UK
Oxford, UK
Philadelphia, USA
Rehovot, Israel
Warsaw, Poland

Cycle V Grantees

Bergamo, Italy
Boston, USA
Chicago, USA
Edgbaston, UK
Hanover, Germany
Heidelberg, Germany
Madison, USA
Minneapolis, USA
Munich, Germany
Nantes, France
Oklahoma City, USA
Toronto, Canada
Toronto, Canada

Cycle VI Grantees

Augusta, USA
Boston, USA
Boston, USA
Brussels, Belgium
Chapel Hill, USA
Durham, USA
Madison, USA
Manchester, UK
Regensburg, Germany
Vienna, Austria

Cycle VII Grantees

Boston, USA
Boston, USA
Brussels, Belgium
Cagliari, Italy
Chicago, USA
Columbus, USA
Milwaukee, USA
Melbourne, Australia
Nantes, France
New York, USA
Pittsburgh, USA

3. Grant Awards in Cycle VII

Dr. Michel Y. Braun, Principal Investigator

Université Libre de Bruxelles, Brussels, Belgium

Mechanisms of Graft Destruction in the Absence of Cognate Recognition between the Graft and T Cells

The majority of alloreactive CD4 T cells directly recognise allogeneic MHC/peptide complexes expressed by the cells of transplanted organs. CD4 T cells can also recognise donor antigens processed by recipient APC and indirectly presented to T cells as peptides in the binding groove of recipient MHC molecules. Though the participation of alloreactive T cells sensitised by the indirect pathway of allorecognition in graft rejection is well documented, their effector function in this process is unclear. It also remains to identify the nature of recipient APC capable of stimulating indirect pathway T cells within the graft and to demonstrate that such interaction is sufficient to promote the destruction of transplanted tissues. Our project will examine the mechanisms that ensure the specificity of tissue destruction in a murine TcR transgenic model where rejection occurs in the absence of cognate recognition between the graft and T cells. More specifically, we shall identify the type of APC capable of stimulating indirect pathway T cells within the graft. APC tested for their stimulatory capacities will include bone-marrow-derived immature dendritic cells, aortic endothelial cells and blood-derived fibrocytes. We shall also study the effector mechanisms of tissue destruction promoted by indirect pathway T cells by using antibody treatment, as well as a genetic approach, to neutralise the function of various lymphokines.

Dr. Anita S. Chong, Principal Investigator
Dr. Dengping Yin, Co-Investigator
Dr. Lianli Ma, Research Assistant
Dr. JiKun Shen, Research Pathologist
Ms. Anncy Varghese, Research Technician

Rush Presbyterian St. Luke's Medical Center, Chicago, USA

Pathogenesis and Protective Activities of Anti-Gal Antibodies

The lack of adequate human organs has created a strong interest in the use of non-human organs for clinical transplantation. However, vigorous immune responses prevent the successful transplantation of non-human organs into humans. In particular, antibodies against a single carbohydrate epitope, Gal-α1,3-Gal, appear to be the major cause of xenograft injury and rejection in the pig-to-primate model. This project focuses on understanding the mechanisms by which anti-Gal antibodies cause graft rejection, and on the definition of novel approaches to control them. We have generated a series of anti-Gal IgG antibodies, and will transfuse these antibodies into mice receiving a rat heart xenotransplant. We will define the immunological and biochemical events that are triggered by anti-Gal antibodies that lead to graft rejection, and attempt to inhibit the rejection by inhibition of specific immunological functions. In addition, we will explore a novel approach of inducing resistance to antibody-mediated injury by pre-treating grafts with low doses of anti-Gal antibodies. We will define the immunological and biochemical events that mediate this resistant state. These studies are directly relevant to the long-term goal of inducing long-term survival of non-human grafts in humans.

Dr. Ezio Laconi, Principal Investigator
Dr. Mario Strazzabosco, Co-Applicant
Dr. Umberto Baccarani, Research Associate
Dr. Bruno Gridelli, Research Associate
Prof. Giuseppe Remuzzi, Research Associate
Prof. Stephen Strom, Research Associate

University of Cagliari, Cagliari, Italy

Towards a Human Hepatocyte-Based Bio-Artificial Liver

Organ transplantation is the only effective treatment for advanced liver disease. However, serious problems, including organ availability and high cost, severely limit its widespread clinical application. As a result, many candidate patients never reach access to this treatment, particularly in case of fulminant hepatic failure, when time is crucial. The possibility of using an external liver support would be lifesaving for many such patients, as confirmed by results of a recent clinical trial. A bio-artificial liver (BAL) can serve at least two main purposes: (i) it can support a patient's life until a donor liver is available, i.e. it can "bridge" to organ transplantation; and (ii) it can promote the recovery of the patient's own liver, thereby avoiding transplantation. Thus, the assembly of a clinically effective BAL system would be highly desirable and is being pursued by several groups of investigators. Ideally, any artificial surrogate of the human liver should include the cells that are normally present in the liver, i.e. normal human hepatocytes. However, current approaches are mostly based on other, less efficient cell types, because it has been difficult so far to obtain sufficiently large numbers of human cells. In this context, we have developed an experimental model that serves as a general strategy for the rapid expansion of transplanted cells in the liver. Our aim is to exploit such a strategy in order to expand human hepatocytes injected into the liver of a host animal. Such a liver, repopulated with human cells, could greatly improve the efficiency of currently available BAL systems. The potential clinical impact would be twofold: (i) more patients with end-stage liver disease would be "bridged" to organ transplant; (ii) more patients with acute liver failure would be helped to recover without transplantation.

Dr. Subramaniam Malarkannan, Principal Investigator
Dr. Jeyarani Regunathan, Research Associate

Medical College of Wisconsin, Milwaukee, USA

Role of Minor Histocompatibility Antigens in Solid Organ Transplantation Rejection

Solid organ transplantation is a vital clinical methodology to save the lives of patients with organ failures due to many physiological etiologies. Historically, organ transplantations and blood transfusion were attempted for hundreds of years. Studies over the past 100 years have made many remarkable milestones in improving acceptance of the graft by the host, one of them being the "matching" of the host and the donor for a set of proteins called Major Histocompatibility Antigens (HLA typing). By matching individuals for HLA physicians can identify donors whose organs potentially will not be rejected by the patient. Acceptance rates of the grafts are significantly higher after matching the donor and the host. However, even after matching a good number of patients reject the transplanted graft. Studies in humans and mice indicated that a second set of molecules, called "Minor Histocompatibility Antigens," is responsible for these rejections. The molecular identities of these molecules are important for understanding the reasons for rejection and to devise ways to improve solid organ transplant acceptance. Our laboratory has successfully identified four of these antigens in mouse systems, two of which are also present in humans. Our studies are focussed to identify, characterize and develop methods for diagnosis (typing for these molecules also before transplantation) and to improve graft acceptance.

Prof. James McCluskey, Principal Investigator
Dr. Jamie Rossjohn, Co-Applicant
Dr. Andrew Brooks, Co-Applicant
Dr. Anthony Purcell, Associate

University of Melbourne, Melbourne, Australia

Defining the Molecular Basis of T cell Allorecognition

Long-term transplant rejection in recipients of kidney and other solid organ transplants is due to an attack on the grafted organ by the T cells of the immune system. This attack results from mistaken identity of the transplanted tissue that resembles an infection to the unsuspecting immune system. The molecular recognition events that occur in transplant rejection are very poorly understood. This research aims to determine exactly how and why this case of mistaken identity occurs at a molecular level.

The project will first determine the three-dimensional structure of several protein receptors that T cells normally use to detect common viral infections but which are also known to attack tissue grafts from unrelated individuals. We will also identify the exact molecules that are recognised on transplanted organs during immune rejection and compare these with the molecules that are normally recognised during viral infections. Once these molecules are identified we plan to determine their shape at the atomic level by X-ray crystallography. By comparing exactly how the same receptors that recognise viruses also recognise foreign tissues we hope to learn more about the basic mechanisms by which T cell receptors interact with host tissues and organ grafts.

The molecular details by which host T cells recognise transplanted tissues may allow us to design ways of monitoring immunological rejection events in transplanted patients and perhaps permit us to design decoy molecules that could subvert the rejection process.

Dr. Simon C. Robson, Principal Investigator
Dr. Imrana Qawi, Research Assistant

Beth Israel Deaconess Medical Center, Boston, USA

Disordered Thromboregulation in Xenotransplantation

Clinical transplantation is limited by the availability of suitable human organ donors. The proposed use of a unlimited supply of animal e.g. pig organs in clinical practice, termed xenotransplantation, could provide a bridge to a later successful human graft in a critically ill patient; or more optimistically, may even substitute for such a graft. Unfortunately, the clinical application of xenotransplantation, to date, has resulted in almost total failure.

Recent developments in the fields of xenotransplantation and blood vessel or vascular biology have greatly expanded our understanding of the mechanisms by which xenografts are rejected and have given new hope to the field. However, one additional novel barrier observed by the Applicant and colleagues is that of molecular incompatibility between the blood vessels of pig organs and primate/human blood coagulation factors and platelets. This causes rapid clotting within transplanted porcine xenografts in baboons with rapid loss. To mitigate against these effects, we propose to derive triple transgenic animals over-expressing two human natural anticoagulants (called tissue factor pathway inhibitor and thrombomodulin) and the thromboregulatory factor CD39, which was found by the Applicant to profoundly inhibit the activation of platelets irrespective of the species of origin. The project proposed in this application is a preliminary component of a long-term clinical xenotransplantation strategy, involving the genetic modification of donor pigs to render them biologically more compatible. We have first checked that the respective human cDNA's can be functionally expressed in porcine vascular endothelium, either individually or in tandem. We now propose derivation of multi-transgenic mice, again checking that there are no deleterious effects and testing function of the over-expressed factors in relevant models of xenotransplantation.

This work will be judged successful if the data result in the testing of novel treatments to prolong xenograft survival and are of potential relevance for clinical transplantation.

Dr. Paul S. Russell, Principal Investigator
Dr. Joren C. Madsen, Co-Applicant
Dr. Robert C. Colvin, Co-Applicant
Mr. Harris S. Rose, Research Associate
Ms. Catharine M. Chase, Research Associate
Dr. Megan Sykes, Collaborator
Dr. David Sachs, Collaborator

Massachusetts General Hospital, Boston, USA

Cellular Mechanisms of Cardiac Allograft Vasculopathy (CAV) in Tolerant and Immunodeficient Mice

These experiments are designed to clarify a new mechanism we have recently discovered for the development of coronary artery disease in transplanted hearts. We were surprised to find that impressive disease can occur in mouse hearts transplanted to incompatible recipients even though they had been made completely inactive ("tolerant") to the known antigens presented by the transplants. Transplants between identical animals never became diseased. Similar results were found with hearts transplanted to mice genetically incapable of any kind of conventional immune response. We feel that so-called "innate" immunity, which can operate quite separately from the usual rejection process, may contribute importantly to this process, a process that seriously limits the survival of many transplanted organs.

The proposed experiments involve performing heart transplants, by microsurgical methods, between special strains of inbred mice. These would include strains in which "natural killer" cells (NK) are inactivated genetically or in which such cells are neutralized by specific antibodies to them. We can also visualize NK, and other important cells, in microscopic sections of the coronary vessels with monoclonal antibodies directed toward them. We hope to define precisely the involvement of "natural killer cells" and certain cytokines in this process and thereby open up new approaches for controlling this pathological process much more effectively.

Dr. Rakesh Sindhi, Principal Investigator
Ms. Amy Magill, Research Technician
Ms. Xiaoting Tang, Research Technician
Dr. Janine Janosky, Statistical Consultant
Dr. Jeff Paslay, Scientific Consultant
Dr. Suren Sehgal, Scientific Consultant

Children's Hospital of Pittsburgh, Pittsburgh, USA

Designing Biomarker-Assisted Clinical Trials for Immunosuppressants

Current evaluation of immunosuppressant agents in transplantation involves the use of fixed doses or concentrations, and non-numeric measures of clinical success or failure. Because nearly half of all subjects enrolled in clinical trials experience some form of failure, e.g. acute rejection or side effects, other ways are needed to evaluate new immunosuppressive drugs. We have previously evaluated several proteins, such as cytokines and cell surface receptors, which mediate lymphocyte responses to transplanted antigens, as potential measures of drug effect. These proteins are sensitive to two commonly used classes of drugs: calcineurin inhibitors such as tacrolimus; and anti-proliferative drugs such as sirolimus and mycophenolate mofetil. Also, their degree of inhibition predicts drug concentrations that are associated with a stable post-transplant course. In the current proposal, our first step will be to identify additional proteins sensitive to immunosuppression with tacrolimus, sirolimus and mycophenolate mofetil in residual human blood samples from subjects enrolled in ongoing studies at our center. One of two high-throughput techniques will allow us to evaluate up to 2000 proteins in plasma and cells with surface-enhanced laser desorption ionization (SELDI)-time of flight (TOF)-mass spectrometry, for potential sensitivity to immunosuppressant agents. Subcellular translocation of proteins or their disruption during proliferation and stimulation will be measured in a 6-channel, fluorescent cell-imaging platform that can focus on the cell membrane, cytoplasm, nucleus, and cytoplasmic organelles. As a second step, protein targets that demonstrate sensitivity to the drugs mentioned will be subjected to effect: concentration modeling to identify thresholds for maximal or sub-maximal effect on protein biomarkers. Within the scope of this effort, a clinical trial design will be proposed at the end of the study period. In this trial, the concentration-dependent, measurable response of biomarkers will be used as an endpoint for drug evaluation instead of non-numeric endpoints. Our prior studies suggest that such a trial would enhance the safety of clinical trial participation and reduce numbers of human subjects needed for drug evaluation.

Prof. Jean-Paul Soulillou, Principal Investigator
Dr. Sophie Brouard, Research Associate
Prof. Robert Lechler, Research Associate
Dr. Fabien Sebille, Research Associate
Ms. Annaïck Pallier, Research Associate
Mr. Marc André Delsuc, Research Associate
Dr. Jean-Christophe Dore, Research Associate
Dr. Minnie Sarwal, Scientific Consultant

Institut de Transplantation et de Recherche en Transplantation (ITERT), Nantes, France

Vβ Transcriptome Regulation during Allograft Rejection and Tolerance

Organ transplantation allows patients with end-stage disease of a vital organ to recover a normal life. Immune recognition of foreign tissues remains the main obstacle to transplantation, which requires long-term immunosuppression. The initial alloimmune responses proceed through the usual pathway of immunity in which allopeptides are presented by self-MHC. However, an unusually high proportion of naive T cells also directly recognise foreign MHC. This "direct" recognition is supposed to play a major role in acute rejection early after grafting. Normal recognition (self-APC) is supposed to operate in chronic rejection.

Our programme deals with a global representation of TCR usages in direct indirect pathways *in vitro* and *in vivo*. So far, TCR biases were only studied through qualitative TCR alteration (Immunoscope) of the Vβ chain segment (CDR3) interacting with peptide/MHC. This is relevant for analysing TCR biases in the indirect but not for direct pathway where CDR3 may not be involved. In addition, in all cases, the possible relevance of qualitative alteration must benefit from the knowledge of the number of clones involved (i.e. reflected by the amount of mRNA).

For the first time, we propose another approach that links qualitative and quantitative parameters and allows a global vision of TCR alterations (TcLand) in which qualitative alterations of Vβ mRNA are corrected by the amount of altered mRNA assessed by quantitative PCR.

Our aims are threefold:

1) To optimise the method.
2) To revisit allorecognition *in vitro*, in "direct type" MLR where pure T cells are confronted with allogeneic APC, and *in vivo* during acute rejection of heart allograft in rat and in one established model of experimental tolerance induction.
3) To identify and purify cells that may be responsible for tolerance in rare cases of human recipients of organ transplants who accept their grafts in the absence of any immunosuppressive drugs.

Dr. Anne M. VanBuskirk, Principal Investigator
Ms. Julie Dierksheide, Research Associate

Ohio State University, Colombus, USA

Cytokine Inhibition of CTL Reactivation and Post-Transplant Lymphoproliferative Disorder

Post-transplant lymphoproliferative disorder (PTLD) is an aggressive B cell malignancy afflicting approximately 2–7% of all transplant patients, with 50–90% mortality. Most PTLDs are B cell lymphomas associated with Epstein-Barr virus (EBV). Primary EBV infection and viral load in the post-transplant period are significant risk factors, but many aspects of PTLD remain poorly defined. Most adults harbor a latent EBV infection controlled by EBV-reactive cellular immune responses, which also prevent PTLD. However, immunosuppressive therapy necessary to prevent graft rejection results in defective immune surveillance, including a drop in EBV-specific CTL activity. The precise mechanism(s) by which the anti-EBV immune response fails is not clear, nor is it clear why only some transplant patients develop PTLD, although immune factors are thought to be important. One key mediator is IFNγ, which contains an A→T polymorphism at residue 847. The T allele is associated with high cytokine production. We recently reported that the A/A genotype for IFNγ is expressed more frequently in PTLD than in non-PTLD patients, and have now confirmed that observation using the SCID-Hu PBL mouse model. These data suggest that low IFNγ is a factor in PTLD. Exciting preliminary data indicate that the A allele of the IFNγ genotype is associated with an ability of TGFβ to inhibit CTL activity. Exogenous IFNγ can overcome this inhibition. We also have data that indicate the inhibition occurs via the APC. We therefore hypothesize that TGFβ induced by immunosuppressive therapy inhibits EBV CTL in PBL with the A/A, but not the T/T, IFNγ genotype, by altering APC function. We are uniquely able to investigate TGFβ-mediated CTL inhibition due to our bank of genotyped PBL donors and HLA-matched tumor cells. These PBL and matched tumor lines will be used to characterize the TGFβ-mediated inhibition and begin to dissect its mechanism. These results will increase our understanding of PTLD by addressing mechanisms of CTL inhibition by TGFβ and the association of CTL inhibition with cytokine genotype. These results may also provide information on potential therapeutic targets to treat or prevent PTLD.

Dr. Liang Zhu, Principal Investigator
Mr. Souvik Sarkar, Research Assistant

Albert Einstein College of Medicine, New York, USA

Targeting Cell Cycle Regulators to Improve Hepatocyte Transplantation Success

Liver transplantation is currently the only effective treatment for patients with terminal liver failure, but severe shortage of donor organs pose great limitations to its use. In the United States alone, more than 25 million people are afflicted with liver and gall bladder diseases and more than 25,000 of them die each year of end-stage liver failure. Yet only 1,500 liver transplantations were performed in the United States in 1994. Clearly, there is an urgent need for effective alternative therapies to treat end-stage liver disease. For very logical reasons, hepatocyte transplantation has long been considered an attractive alternative to liver transplantation. A major obstacle to the successful use of hepatocyte transplantation is that the number of hepatocytes that can be transplanted is too small to significantly impact the course of the diseases being treated.

Proliferation of hepatocytes, like all other types of cells in the body, is controlled by a group of proteins collectively called the cell cycle regulators, which either positively or negatively regulate cell proliferation. Recently, our laboratory set out to investigate the effects of inactivating a negative cell cycle regulator (called p27Kip1) on the proliferation of hepatocytes after their transplantation. Using mice as animal models, we have shown that hepatocytes deficient in p27Kip1 proliferated better in host livers and achieved better efficiency in rescuing host liver failure. This finding identified a novel approach to improve the success of hepatocyte transplantation. In the current proposal, we will use new mouse experimental systems that more closely resemble human hepatocyte transplantation conditions to determine the clinical potential of this new concept of targeting cell cycle regulators to improve hepatocyte transplantation success.

4. Progress Reports of ROTRF Grantees

Prof. David Adams, Principal Investigator
Dr. Sarah Goddard, Co-Applicant

University of Birmingham, Birmingham, UK

Human Hepatic Dendritic Cells Induce Tolerance via Notch Signalling

Characterisation of human liver dendritic cells

The liver is associated with relatively less aggressive immune responses than other tissues; for instance, liver allografts are more easily accepted, with a lower rate of rejection than is seen for other solid organ allografts. In addition the liver may be seen as a carrier organ for some viruses, such as hepatitis C and B, that may lead to chronic infection.

We concluded that dendritic cells (DCs) in the liver may be responsible for regulating liver-specific immune responses, and we have therefore looked at the phenotype and function of dendritic cells isolated from human liver. Recently a number of new antibodies have become available, allowing a more thorough analysis of phenotype. Immunohistochemistry of normal human liver tissue shows expression of both DC-SIGN and CD123 in cells with the morphology of dendritic cells in the portal tracts. However there is no expression of the plasmacytoid dendritic cell marker BDCA-2.

Immunocytochemistry and flow cytometry of cells isolated by overnight migration from normal human liver confirms that human liver DCs express DC-SIGN and low levels of CD123. The CD123 expression was detected on class II positive cells, and on further analysis a proportion of these cells were found to be negative for CD14. The cells were also negative for BDCA-2. Recent publications suggest that there is a human dendritic cell subset, which expresses CD11c, with low level CD123 in peripheral blood (Dzionek *JI* 2000). We therefore investigated the marker for this subset, CD1c/BDCA-1, and found that this marker was expressed in all liver-derived DCs.

Function of human liver dendritic cells

In order to assess the function of the DCs, a number of approaches have been used.

1) Investigation of the expression of a range of cytokines by DCs, using a fluorescent micro-particle immunoassay (Luminex 100) that is able to accurately quantify many molecules from small volumes of culture media.

2) Extension of earlier studies of IL-12 production by DCs, using real-time PCR to assess the control of expression of the IL-12 subunits.

3) Investigation of the expression of cytokines by T cells in coculture with allogeneic DCs using intracellular cytokine staining and flow cytometry.

Luminex cytokine quantification was done using media from dendritic cell cultures. Liver- and skin-derived cells were studied, in addition to monocyte-derived DCs. Nineteen different molecules were assessed; of these IL-8, IL-6 and IL-1 were found in both liver- and skin-derived dendritic cell cultures, at all time points and in all conditions. Eotaxin, IL-12, IL-2, IL-4, IL-5, IL-13, SDF-1 and IL-7 were not detected. MCP-1 was detected in liver dendritic cell conditioned media, but not in media from skin- or monocyte-derived DCs. Other cytokines such as TNF-α and IL-15 were present in some cultures, but their expression was more dependent on conditions of dendritic cell stimulation, than on the source of the dendritic cells (Table 1). Further studies are planned to clarify these findings.

	MCP-1	RANTES	TNF-α	IL-15	IL-10
Liver DCs stim	+	+	+	-	+
Liver DCs unstim	+	-	-	+/-	+
Skin DCs stim	-	-/+	+	Low	+
Skin DCs unstim	-	-	-	Low	-
Monocyte DCs	-	+	-	+	+

Previous work has shown that IL-12p70, the active form of the cytokine, is not produced by liver-derived DCs, even after stimulation with CD40L. Stimulation with CD40L, as well as LPS and IFNγ, has now been investigated, thus providing the DCs with a T cell, and a bacterial stimulus, which is thought to be important for IL-12p70 production (Schulz *Immunity* 2000). It was again found that IL-12 is not produced. Many publications in this area report expression of IL-12p40, one of the subunits. It was found that the gene for this subunit is expressed. Real-time PCR is now being used to quantify gene expression of the subunits.

In addition to investigating the production of cytokines by DCs, the function of DCs in T cell coculture has also been studied. DCs were cocultured with pure allogeneic T cells, from cord blood or adult peripheral blood for five days, and then re-stimulated. The T cell response stimulated by the DCs was then assessed by intracellular cytokine staining of the cells for IL-2, IL-4, and IFNγ. The staining was detected on T cells using dual colour flow cytometry. A high proportion of the skin dendritic cell-stimulated T cells were found to express IFNγ, and there was less expression of IFNγ by liver dendritic cell-stimulated T cells, and additionally, these cells expressed IL-4 and IL-10 (Figure 1).

Role of Jagged and Notch in dendritic cell function

Preliminary data obtained in collaboration with Jonathon Lamb's laboratory in Edinburgh led us to investigate the role of Jagged and Notch in the generation of pro-tolerance responses by dendritic cells. We have now set up real-time PCR in Birmingham to continue these studies.

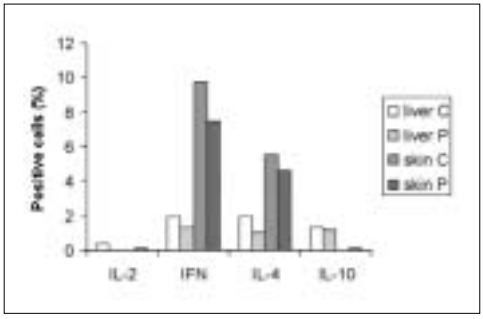


Figure 1. *Cord blood T cells (unshaded "C") and peripheral blood T cells (cross-hatched "P") were cocultured with dendritic cells from liver (white bars) or skin (grey bars) for 5 days and then restimulated with plate-bound antibody to CD3 and CD28 in the presence of monensin. The cytokine produced was then stained and T cell expression of cytokine assessed by dual colour flow cytometry. The percentage of positive cells compared with control cells is shown. Skin dendritic cell-stimulated cells produce most IFNγ, whilst liver dendritic cell-stimulated T cells produce most IL-10.*

Initially we found that the conventional method for isolating RNA, using phenol/ chloroform, allowed some transfer of genomic DNA. We have therefore switched to the Quiagen system, which incorporates a DNAase step, and which has proved successful. Coculture experiments are now being established to collect RNA for testing, and we are assessing whether archived material can be used in the Quiagen system. Full analysis of Jagged and Notch expression in different *in vitro* models will then be done, using the liver DCs.

The results so far show that liver dendritic cells conform to a 'myeloid' phenotype, with expression of CD11c, with only low levels of CD123, and no expression of BDCA-2 (both markers of 'plasmacytoid' type DCs) (Figure 2). However their cytokine expression is consistent with the generation of less aggressive immune responses. IL-10 is involved in regulatory T cell generation, and it has recently been suggested that MCP-1 may be involved in Th2 responses (Cyster *Nat Imm* 2002). In support of this hypothesis we find that T cells stimulated by liver dendritic cells produce a pattern of cytokine expression more commonly associated with Th2. We will now go on to confirm these initial findings and to define in more detail the immune reponses stimulated by human liver DCs.

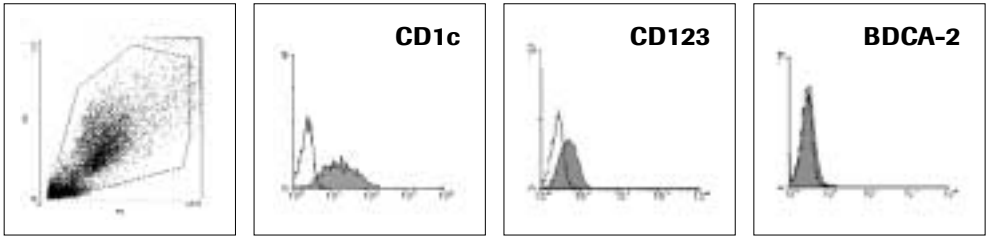
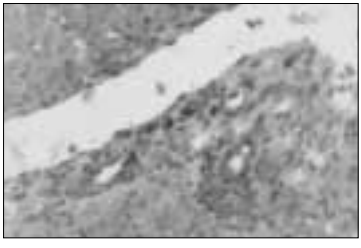


Figure 2. *Flow cytometry of liver-derived dendritic cells shows that these dendritic cells express CD1c and some CD123, but are negative for BDCA-2. Histology of normal liver confirms that dendritic cells in the portal tracts express CD123 (IL-3R), as well as some endothelial cells.*



Dr. Simi Ali, Principal Investigator
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Anti-Rejection Therapy: Modifying Intragraft Immunity by Specific Blockade of Th1 Cell Recruitment

Acute transplant rejection is dependent on the pattern of leukocyte migration and activation. The movement of selected leukocyte sub-populations from the blood, across the endothelial cell monolayer and basement membrane and into the underlying tissue is an important component of an inflammatory response. The control of the recruitment of specific leukocytes to inflammatory sites therefore involves the concerted action of various combinations of selectins, chemoattractants and integrins. It is the multifactorial nature of this process that results in selectivity *in vivo.*

The chemokines, a family of small chemoattractant proteins, play a role in activation and directional migration of specific leukocyte populations. The chemokines interact with a family of 7-transmembrane-spanning G-protein-coupled receptors; in addition they also interact with cell surface glycosaminoglycans (GAG). It has been demonstrated *in vitro* that different chemokines have differential selectivity for GAG families. Based on the selectivity of a particular chemokine for the different GAGs, it is likely that different chemokines will bind with differing affinities, or may not bind at all, to the GAGs present on the vascular endothelium during a particular inflammatory condition. To conclude, chemokine function during inflammatory responses may depend both on interactions with its specific receptor and lower affinity interactions with abundant GAG molecules such as HS. In order, to provide a strategy for immune regulation it is important to understand how these interactions define precisely the selectivity of chemokines.

Work carried out so far: The first year of this study has sought to investigate the specific role of GAGs in inflammation. With endothelial cells (HMEC-1) using flow cytometry and confocal microscopy we demonstrated that heparan sulphate is most abundantly expressed on these cells followed by chondroitin-4-sulphate, chondroitin–6-sulphate and dermatan sulphate, respectively. Regulation of the biosynthesis of heparan sulphate (HS) and the relationship between expression and structure/function is complex and little understood. We hypothesised that in response to inflammatory factors such as cytokines the vascular endothelium modifies its GAG component to change the biological state. This change then enables the endothelium to sequester pro-inflammatory factors such as the HS binding CC and CXC chemokines in order to direct the inflammatory response: for example, promoting transendothelial migration of lymphocytes.

We stimulated HMEC-1 cells with pro-inflammatory cytokines IFN-γ (100 IU/ml) and TNF-α (100 IU/ml) for 24 h. These cells were then overlaid with biotinylated-RANTES and subsequently with fluorescein-avidin according to manufacturer's instructions (R&D Systems Inc). Relative amounts of biotinylated chemokine bound to the GAGs were quantified by confocal microscope. These cells showed an increased capacity to bind chemokine compared to untreated cells. To verify the specificity of this interaction, cells were treated with chlorate (inhibitor of GAG biosynthesis) and heparitinase (enzyme specific for heparan sulphate) which resulted in reduced binding of exogenous RANTES.

In addition, we used normal and rejecting paraffin embedded renal biopsy sections for localisation of GAGs and their capacity to bind chemokines. Using confocal microscopy it was found that the expression of heparan sulphate, chondroitin sulphate and dermatan sulphate was significantly different between normal and rejecting samples, with heparan sulphate being the most abundantly expressed of the three GAGs. The distribution of heparan sulphate was largely basolateral of renal tubules with some expression on the apical surfaces in the normal kidney, whereas in rejecting kidney it was only on basolateral surfaces. Chondroitin sulphate was mostly restricted to interstitial cells in both normal and rejecting sections. The GAG-specificity of RANTES binding is currently under investigation, using Abs specific for prototypical CC chemokine RANTES. The increased potential of cell surface GAGs to bind chemokines in rejecting tissue may provide a regulatory mechanism to increase infiltration during inflammation (Oral presentation Chemokine Conference 2002, Madrid, Spain).

We had earlier created a panel of mutant RANTES molecules containing neutral amino acid substitutions within putative, basic GAG binding domains. We have now analysed these mutants and found that, despite showing reduced binding to GAGs, each mutant containing a single amino acid substitution induced similar leukocyte chemotactic response, within a concentration gradient generated by free solute diffusion. However, it was found that the mutant K45A had a significantly reduced potential to stimulate chemotaxis across a monolayer of microvascular endothelial cells. Significantly, this mutant showed a potential to mobilise Ca^{2+} and bound to the CCR5 receptor with an affinity similar to the wild type (wt) protein. We further investigated the requirement of GAG binding for apical presentation of chemokine and its role in monocyte trans-endothelial migration (TEM). We compared the relative potential of wt and K45A RANTES to promote TEM. RANTES immobilised on the apical surface could promote limited TEM across endothelial cells. In comparison, the TEM induced by K45A was significantly lower. These results show that the interaction between RANTES and GAGs is not necessary for specific receptor engagement, signal transduction or leukocyte migration. However, this interaction is required for the induction of efficient chemotaxis through extracellular matrix between confluent endothelial cells (Ali S *et al.* Manuscript submitted).

Studies are underway to analyse the capacity of K45A to induce adhesion and trans-endothelial migration compared with wild-type RANTES under shear flow in collaboration with Prof. David Adams (MRC Liver Unit, Birmingham, UK).

Publications

Ali A, Malik G, Robertson H, Kirby JA. Inflammation increases N sulphation and the chemokine binding potential of cell-surface glycosaminoglycans. Abstract chosen for oral presentation at Chemokine Conference 2002, Euroforum El Escorial, Madrid, Spain (May 5-9, 2002).

Ali S, Fritchley SJ, Chaffey BT, Kirby JA. Contribution of the putative heparan sulphate binding motif BBXB of RANTES to trans-endothelial migration. Manuscript submitted.

Prof. Reza Dana, Principal Investigator
Prof. Andrius Kazlauskas, Co-Applicant
Dr. Ying Qian, Research Associate
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Promotion of Corneal Transplant Survival by Anti-Apoptotic Genes

I. Localization of green fluorescent protein (GFP) gene expression mediated by adenoviral vector in corneal grafts *in vivo*

METHODS: BALB/c mice aged 8 to 10 weeks were used as corneal donors and recipients. All animals were treated in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. BALB/c corneas were incubated with 6 x 10^7 pfu of adeno-GFP vector or adeno-empty vector *ex vivo*, in tissue culture plates for 2 hours at 4°C. The GFP adenoviral vector was an E1- and E3-deleted adenovirus carrying the GFP gene that was constructed, propagated in 293 cells, purified by density gradient method, and stored at -80°C until use. Both GFP adenoviral and control ('empty') vector (containing no GFP insert), were provided by the Harvard Gene Therapy Initiative. The donor BALB/c corneas infected with GFP adenoviral vector or control empty vector were transplanted orthotopically into BALB/c host corneal beds with 8 interrupted 11-0 nylon sutures. One week after surgery, the expression of GFP in the corneal grafts was examined in cryosections of the eyeballs bearing the syngeneic corneal grafts using an epifluorescent microscope.

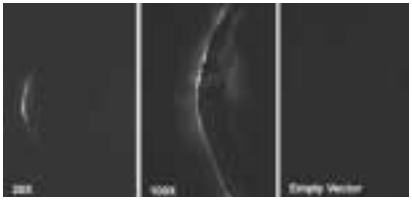

Figure 1. *Localization of GFP in the endothelium (arrows) of corneal syngeneic grafts. Empty adenoviral vector served as a control.*

RESULTS: GFP expression was found to be almost entirely restricted to the corneal endothelium layer of the corneal syngeneic grafts, with only minimal expression in the stroma. There was no GFP expression noted in the corneal epithelium (Figure 1).

CONCLUSION: Adenoviral vector selectively and efficiently delivers exogenous gene to the endothelium of corneal grafts.

II. Adenoviral vector-mediated delivery of GFP marker gene to syngeneic corneal grafts

METHODS: BALB/c mice aged 8 to 10 weeks were used as corneal donors and recipients. BALB/c corneas (n = 4 /group) were incubated with 6 x 10^7 pfu of adeno-GFP vector or

adeno-empty vector *ex vivo* in tissue culture plates for 2 hours at either 4°C or 37°C, the former is a temperature commonly used for human donor cornea storage. The BALB/c donor corneas infected with GFP adenoviral vector or control empty vector were transplanted orthotopically into BALB/c host corneal beds with 8 interrupted 11-0 nylon sutures. After surgery, the kinetics of GFP expression in the corneal grafts were evaluated *in vivo* using epifluorescent microscopy, and the clinical outcome of the grafts was evaluated using slit-lamp biomicroscopy. At each time point, grafts were scored for opacification using a previously described and standardized scoring system which grades the opacification of corneal grafts.

RESULTS

1. Kinetics of GFP Expression: With an incubation temperature of 4°C, *in vivo* expression of GFP in syngeneic corneal grafts was demonstrated for up to 12 weeks with peak expression from day 3 to week 5 (Figure 2). With an incubation temperature of 37°C, GFP expression in the corneal grafts lasted less than 1 week (Figure 3). Syngeneic grafts incubated with the vector at 4°C exhibited a larger area and longer expression of green fluorescence than grafts incubated at 37°C (Figures 4 and 5). The results demonstrate that incubation at 4°C is a better temperature at which to prolong GFP expression in corneal transplants *in vivo*. This is fortunate, since 4°C is the standard temperature for maintaining donor tissue prior to clinical transplantation, so that, theoretically, the adoption of such gene therapy strategies by eye banks should not require too many modifications to their current practices.

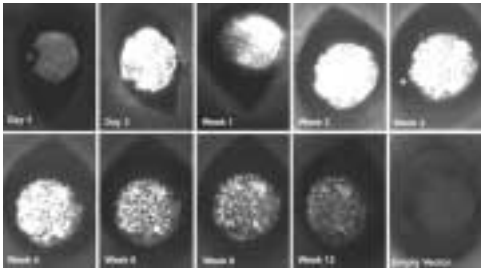

Figure 2. *GFP expression in a representative corneal syngeneic graft infected with adeno-GFP vector ex vivo at 4° C. Empty adenoviral vector served as a control.*

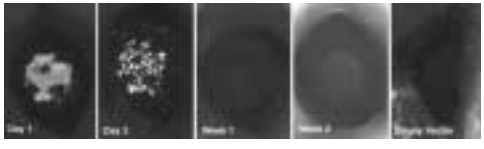

Figure 3. *GFP expression in a representative corneal syngeneic graft infected with adeno-GFP vector ex vivo at 37° C. Empty adenoviral vector served as a control.*

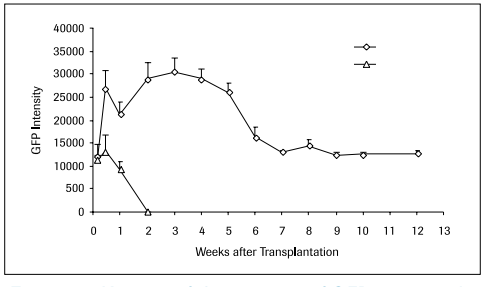

Figure 4. *Kinetics of the intensity of GFP expressed in corneal syngeneic grafts infected with adeno-GFP vector at 4° C and 37° C.*

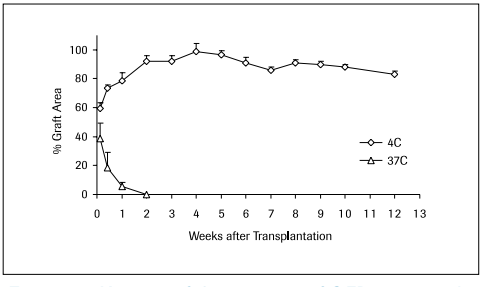

Figure 5. *Kinetics of the intensity of GFP expressed in corneal syngeneic grafts infected with adeno-GFP vector at 4° C and 37° C.*

2. Clinical Outcome: During the first week after transplantation, grafts infected with adeno-GFP at 4°C exhibited an opacity score of 2.6 ± 0.4, which represented inflammatory responses to surgical trauma. After removal of corneal sutures at week 1, the opacity score dramatically decreased and the grafts became clear and maintained their transparency up to week 4 (Figures 6 and 7A). However, many of the grafts developed epithelial degenerative changes during week 4 to week 8 which is probably due to repeated anesthesia and exposure to fluorescent light of 480 nm wave length (similar changes had been seen in other eyes that had not been exposed to any viral vector but had undergone a similar extensive exposure to light). After week 8, the epithelial lesions healed spontaneously and left scars in the corneal grafts (Figures 6 and 7B).

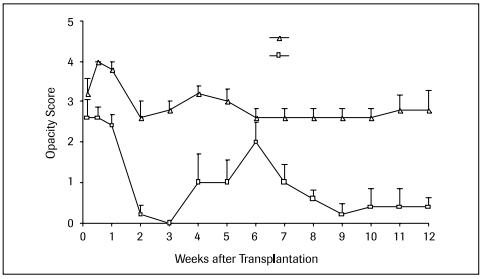

Figure 6. *Clinical opacity scores of the adeno-GFP infected corneal syngeneic grafts.*

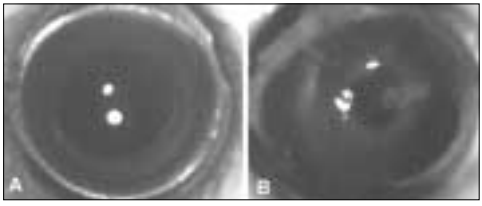

Figure 7. *Gross appearance of the adeno-GFP infected corneal syngeneic grafts at week 4 (A) and week 8 (B).*

The grafts infected *ex vivo* at 37°C, similar to those at 4°C infection conditions, also exhibited an early inflammatory response during the first week after transplantation. The opacity score, however, was higher than that at 4°C. After suture removal at week 1, the grafts remained opaque, with opacity scores ranging from 2 to 3 (Figure 6).

CONCLUSIONS: The expression of GFP gene is retained *in vivo* in corneal syngeneic grafts for prolonged periods of time. An incubation temperature of 4°C is more effective for infection to prolong GFP expression in the corneal transplants *in vivo*, than 37°C.

III. Adenoviral vector-mediated delivery of green fluorescent protein (GFP) marker gene to allogeneic corneal grafts

METHODS: BALB/c and C57BL/6 mice aged 8 to 10 weeks were used as corneal recipients and donors, respectively. BALB/c corneas ($n = 4$ per group) were incubated with 6 x 10^7 pfu or 6 x 10^6 pfu of adeno-GFP vector or adeno-empty vector *ex vivo* for 2 hours at 4°C. The C57BL/6 donor corneas infected with GFP adenoviral vector or control empty vector, were transplanted orthotopically into BALB/c host corneal beds with 8 interrupted 11-0 nylon sutures. After surgery, all grafts were evaluated for *in vivo* expression of GFP, using epifluorescent microscopy, and for signs of rejection using slitlamp biomicroscopy.

RESULTS: Corneal allogeneic grafts infected with a high dose (6 x 10^7 pfu) of the viral vector, exhibited transient GFP expression (Figure 8), whereas the grafts infected with a low dose (6 x 10^6 pfu) of the vector displayed longer GFP expression for up to 3.5 weeks (Figure 9). Clinically, 100% corneal allograft infected with the vector at a high dose failed at week 1, whereas the grafts infected with the vector at a low dose survived until week 3.5. Control grafts infected with empty vector survived for 4–5 weeks (Figure 10), but the rate of rejection was still more rapid than historic controls not using any vector.

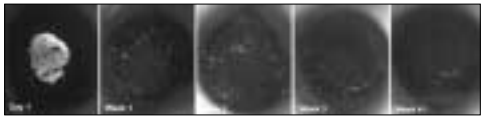

Figure 8. *GFP expression in representative corneal allogeneic grafts infected with adeno-GFP vector at a dose of 6 x 10^7 pfu.*

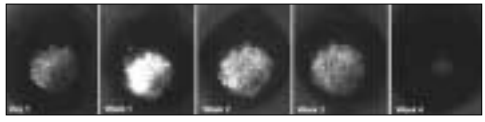

Figure 9. *GFP expression in representative corneal allogeneic grafts infected with adeno-GFP vector at a dose of 6 x 10^6 pfu.*

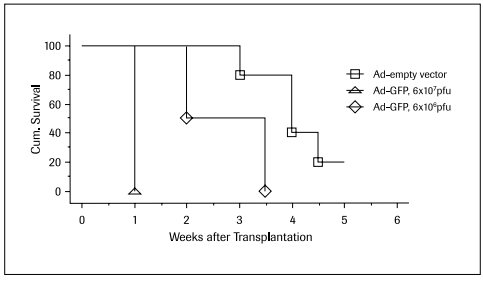

Figure 10. *Kaplan-Meier survival curves of the corneal allogeneic grafts infected with adeno-GFP vector at a dose of 6 x 10^6 pfu or 6 x 10^7 pfu. Grafts infected with empty adenoviral vector or no vector served as controls.*

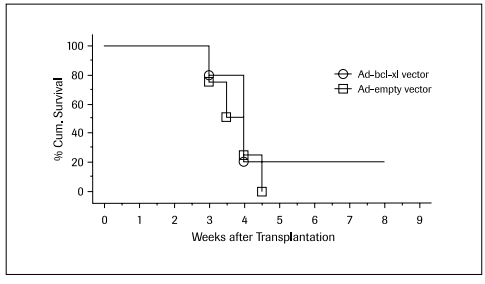

Figure 11. *Survival of adeno-bcl-xl vector or ad-empty vector infected corneal allografts (B6 to BALB/c).*

CONCLUSION: GFP gene expression, mediated by high-dose adenovirus in the setting of corneal allotransplantation, is short-term and toxic to grafts. Reduction in viral dose prolongs the transgene expression and delays the onset of vector induced graft failure. However, the desired gene expression can only be retained *in vivo* in corneal allogeneic grafts for shorter periods of time than in syngeneic grafts, suggesting that the cumulative burden of allogeneic stimulation and (toxic) adenoviral load is deleterious to the corneal allograft. Additional work needs to be performed to identify optimal conditions for adenoviral vector-mediated gene expression in corneal allografts.

IV. Adenoviral vector-mediated delivery of anti-apoptotic gene (bcl-xl) to allogeneic corneal grafts

We performed experiments using bcl-xl transgenics that overexpress this important anti-apoptotic member of the bcl-2 gene superfamily, regulated by a neuron-specific promoter.

We demonstrated that the expression of bcl-xl in the corneal endothelium (which is neural crest-derived) is significantly elevated as compared to wild-type controls without the transgene. Experiments were then performed where either transgenic donors over-expressing this anti-apoptotic factor ($n = 10$), or wild-type donors ($n = 10$) were grafted onto fully allogeneic hosts. Kaplan-Meier survival analysis showed that donor tissue, over-expressing bcl-xl, shows a profoundly enhanced rate of survival as compared with wild-type donors ($p = 0.04$), providing support for the hypothesis that overexpression of anti-apoptotic genes can be protective for graft survival. Based on these data, we proceeded to evaluate the efficacy of *ex vivo* adenoviral-mediated targeting of corneal endothelial cells for transfer of bcl-xl gene in the context of corneal transplantation.

METHODS: BALB/c and C57BL/6 mice aged 8 to 10 weeks were used as corneal recipients and donors, respectively. BALB/c corneas ($n = 4$ per group) were incubated with 6 x 10^6 pfu of adeno-bcl-xl vector, or adeno-empty vector *ex vivo* in tissue culture plates for 2 hours at 4°C. The C57BL/6 donor corneas, infected with GFP adenoviral vector or control empty vector, were transplanted orthotopically into BALB/c host corneal beds with 8 interrupted 11-0 nylon sutures. After surgery, the grafts were evaluated for signs of rejection by using slitlamp biomicroscopy, and for *in vivo* expression of bcl-xl by using immunostaining.

RESULTS: The adeno-bcl-xl infected corneal allografts exhibited the same rate of rejection as the adeno-empty vector infected corneal allografts. By the middle of week 4 (4.5 weeks), 100% of the grafts in adeno-bcl-xl infected group had been rejected, and 80% of the grafts in the controls had been rejected (Figure 11). Grafts in both groups survived for significantly shorter periods of time compared with conventional, non-vector-infected corneal transplants.

CONCLUSIONS: In contrast to the data we derived using transgenic tissues, under the viral vector gene transfer conditions we have used thus far, adeno-bcl-xl does not prolong the survival of corneal allografts. Additional work needs to be performed to identify possible causes of these results.

DISCUSSION:

The results derived thus far can be summarized as follows:

- *In vitro* and *in vivo* work to date demonstrate the feasibility of adenoviral-mediated gene transfer to the corneal endothelium *ex vivo*. Indeed, the adenoviral vector can selectively and efficiently deliver exogenous gene to the endothelium of corneal grafts during hypothermic (4°C) organ preservation.

- Additionally, the desired gene expression is retained *in vivo* in corneal syngeneic grafts for significantly longer periods of time than has been reported for other tissues. However, retention of the gene product expression is of a much shorter duration in the allogeneic, compared with the syngeneic graft setting.

- It appears that adenoviral gene transfer, with or without GFP expression, has a toxic effect on the corneal endothelium. Thus, whereas transgenic allogeneic donor tissues, over-expressing certain anti-apoptotic gene(s) (e.g. bcl-xl), have a prolonged survival after transplantation, it has not to date been possible to demonstrate this, using viral vector mediated gene therapy approaches.

The potential reasons for this are many:

- Is there inadequate expression of gene product after adeno infection?
- Is there too much redundancy in the apoptotic pathways, such that over-expression or under-expression of one or other factors does not have a significant effect on the final outcome? This is unlikely to be the main reason given the positive data using transgenic donors.
- It is possible that the endpoints measured in this project (revolving around graft survival) are too global, and perhaps not adequately sensitive to modest changes in apoptosis at the level of the corneal endothelium.

Continuation of this project will be through additional *in vitro* and *in vivo* experiments. In the former, the relevance of a number of apoptotic factors in standard assays of apoptosis will be tested, using cultured corneal endothelial cells. This will allow better identification of potential targets for therapy. Additionally, immunosuppressives will be used for a short period in the post-operative period only, and this therapy will then be withdrawn in order to assess whether adenoviral transfer of desirable genes can then exhibit enhanced graft survival. Our hypothesis is that suppression of the anti-viral immune response, for a brief period after surgery, will be adequate for reducing the toxicity of this efficient vector system, so as to prevent graft survival even after termination of the immunomodulatory treatment.

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Dr. Gary Levy, Co-Applicant

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Interaction of OX2 with its Receptor Controls Organ Rejection

Induction of tolerance is crucial for promoting long-term graft acceptance. Previous studies from our group established that infusion of alloantigen into the portal vein (pv) decreased graft rejection, in association with altered cytokine production and increased expression of CD200 (previously referred to as OX2) on dendritic cells. Anti-CD200 mAbs decreased, while the immunoadhesin, CD200Fc, prolonged graft survival. CD200 immunoregulation follows interaction with $CD200R^{+}$ cells.

We have hypothesized that optimal regulation, to decrease transplant rejection, will come from some combination of inhibition of functional expression of costimulator molecules CD80/CD86/CD40, together with facilitation of CD200:CD200R interaction, to promote tolerance. In the grant application (subsequently funded by ROTRF), we proposed several proof-of-principle studies to explore this hypothesis, making use of two strains of mice currently being developed, a $CD200^{tg/tg}$, and a CD200R knockout strain.

The group at DNAX has documented the phenotype of a CD200 KO mouse, finding a predisposition to autoimmune disorders (EAE and arthritis)[1], as predicted. It was also predicted that organs from a $CD200^{tg/tg}$ mouse would be less readily rejected, though increased survival would be blocked by a Fab anti-CD200R mAb. We predicted that a CD200R KO mouse was also predicted to share the autoimmune phenotype of the CD200 KO, but that the expected reduction in allograft survival in the CD200R KO would not, unlike in the CD200 KO, be prevented by an infusion of CD200Fc.

Further characterization of immunoregulation mediated by CD200Fc and anti-CD200R

We have continued to make progress with characterization of immunoregulation using these reagents, anti-CD200R and CD200Fc. Two manuscripts are in press which document our more recent findings.

Preliminary work on construction/characterization of a CD200tg/tg mouse

Mice: Male C57BL/6 mice, and NMRI breeder mice carrying a reverse tetracycline regulated transactivator gene (TgN(rtTAhCMV)4Uh-abbreviated hereafter as $rtTA^{tg}$), were purchased from the Jackson laboratories, Bar Harbour, Maine. Mice were housed five per cage, and allowed food and water *ad libitum*. All mice were used at 8-12 weeks of age. Genotyping of progeny from further backcrosses of $rtTA^{tg}$ to C57BL/6 mice used primer pairs designed in

accordance with the Jackson laboratories' instructions (see below and Figure 1a). The primer pairs used for characterization of these mice were as follows:

Control primer pairs for tTA transgene; amplifies 200bp fragment from wild-type allele
Tcrd primer: 5'CAAATGTTGCTTGTCTGGTG3'

Tcrd antisense primer: 5'GTCAGTCGAGTGCACAGTTT3'

Primer pairs for tTA transgene; amplifies 450bp fragment from the tTA portion of transgene
Tet sense primer: 5'CGCTGTGGGGCATTTTACTTTAG3'

Tet antisense primer: 5'CATGTCCAGATCGAAATCGTC3'

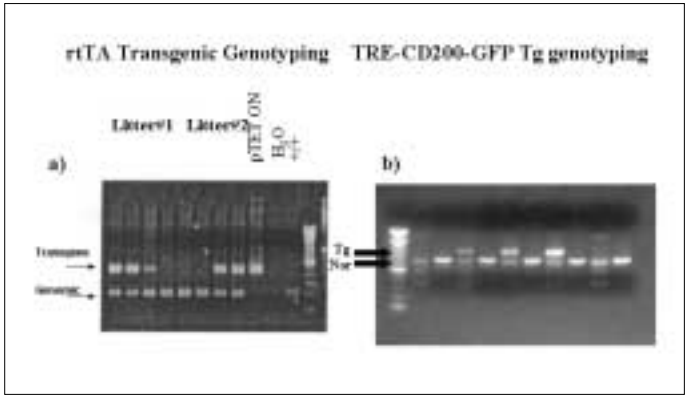

Figure 1. *Analysis of transgenic expression of rtTA (panel a) and TRE-CD200-GFP (panel b) in mice back-crossed three generations to C57BL/6. Expression of endogenous CD200 was detected using the same 5'primer and a 3' antisense primer from the 5' end of the intron immediately downstream of exon 3 (V-region exon of CD200). Housekeeping genes for rTA mice were prepared according to the breeder's instructions (Jackson Labs).*

Creation of TRE-CD200-GFP cDNA, and characterization of transgenic mice: cDNA encoding green fluorescent protein (GFP) was cut from pEGFP-N2 and linked in frame immediately downstream of a tetracycline response element (TRE) gene, and verified by sequencing from a $PTRE_2$ vector using primer pairs (5' ACATGAATTTTACAATAGCG-3') and GFP primer (5'-AACCGTCAGATCGCCTGGAG-3'), flanking the cloning sites in the vector. Sequences were analyzed using the DNAsis for windows, sequence analysis software. The CD200 gene was ligated into this TRE-GFP construct using SacII and BamH1 sites. Confirmation of in frame ligation of TRE-CD200-GFP was made by sequencing using the same primer pairs as described above.

In preliminary studies, we confirmed that co-transfection of CHO cells with both the TRE-CD200-GFP cDNA and rtTAhCMV cDNA led to a doxycycline-inducible increase in both GFP (assessed by fluorescence) and CD200. This was as determined by western analysis of extracts of transfected, doxycycline-induced cells, analysed using a previously described anti-CD200 mAb[2]. Using CHO cells transfected with this TRE-CD200-GFP construct, and stimulated with doxycyline, we verified that surface detection by the anti-CD200 mAb was still apparent, and that the transfected CHO cells caused inhibition of MRL reactivity, as previously described for CHO cells transfected with CD200 alone (see[3]).

Purified TRE-CD200-GFP cDNA was used to transfect embryonic stem cells (University Health Network Transgenic facility). The embryos were transferred to pseudopregnant foster mothers, and subsequent progeny typed for wild-type and transgenic CD200 using the same forward primer (from the V-region exon of CD200) and 3' primer pairs, to distinguish the endogenous germline CD200 and the transgenic CD200-GFP genes as follows:

Sense primer for both endogenous and transgenic CD200 (from V-region exon 3):
5'- GAAGTGGTGACCCAGGATGA -3'

Antisense primer for endogenous CD200 (from 5' end of the intron immediately downstream of exon 3):
5'-TGCTGGCTGTACCCTTAGAA-3'

Antisense primer for transgenic CD200-GFP (from 3' end of GFP cDNA):
5'-TCGTGCTGCTTCATGTGGTC-3'

Of 25 progeny screened (9 males and 16 females) positive transmission of CD200-GFP was detected in 15 (4 males and 11 females), and germline transmission in six (2 males and 4 females). Typical PCR data showing detection of germline and transgenic CD200 in representative mice are shown in Figure 1b. First generation transgenic progeny were backcrossed onto a C57BL/6 ($H2^{b/b}$), typed at each generation, and three founder lines of each continued through further backcross generations with C57BL/6. We are currently breeding to produce 4th generation mice. At the 3rd backcross transgenic (F3) mice were crossed with rtTAhCMV transgenic mice, offspring typed for both rtTA and CD200-GFP transgenes, and positive progeny (referred to subsequently as $F1_3$s) used for *in vitro* analysis as described below.

Doxycyline induction of GFP and CD200 expression in $F1_n$ mice: Peripheral blood cells (PBL) were obtained, using heparinized tubes, from the tail of doubly transgenic $F1_3$ mice (~100µl of blood/donor), before and after allowing mice to drink plain water or water with 2mg/ml doxycycline hydrochloride with 5% sucrose (Dox-water), for a minimum of three days. Fresh water or Dox-water was used at three day intervals. Pre- and post-induction PBL were spun over mouse lymphopaque and examined by FACS for fluorescence (GFP). In addition, an aliquot of cells was incubated

Induction of CD200 expression in fibroblasts of F1 matings from tTA and TRE-CD200-GFP transgenic mice after exposure to doxycycline

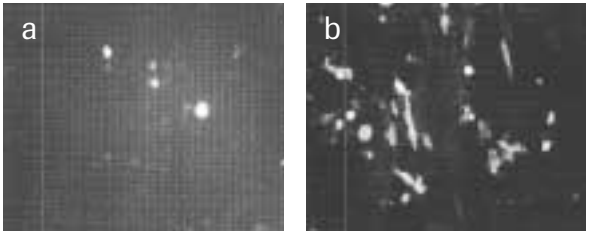

Figure 2. *Induction of GFP expression in fibroblasts (tail skin) prepared from $F1_3$ cross of $rtTA^{tg}$ and TRE-CD200-GFP^{tg} mice, after incubation of fibroblasts in the presence (panel b) or absence (panel a) of doxyxcyline.*

with FITC-labeled anti-CD200 and again examined by FACS. Data in Figure 2 show GFP expression in fibroblasts obtained from $F1_2$ mice, cultured in the presence or absence of doxycyline.

Monoclonal antibodies: The following monoclonal antibodies (mAbs) were obtained from Pharmingen unless otherwise stated: anti-IL-2 (S4B6, ATCC; biotinylated JES6-5H4); anti-IL-4 (11B11, ATCC; biotinylated BVD6-24G2); anti-IFNγ (R4-6A2, ATCC; biotinylated XMG1.2); anti-IL-10 (JES5-2A5; biotinylated, SXC-1); anti-IL-6 (MP5-20F3; biotinylated MP5-32C11); anti-TNFα (G281-2626; biotinylated MP6-XT3). FITC anti- CD80, FITC anti-CD86, FITC anti-CD40, FITC anti-αβTCR, L3T4 (anti-mouse CD4), anti-thy1.2 and anti-Ly2.2 were obtained from Cedarlane Labs. The hybridoma producing DEC205 (anti-mouse dendritic cells) was a kind gift from Dr. R. Steinman, and was labeled directly with FITC; unconjugated and FITC-conjugated rat anti-mouse CD200 was obtained from BioCan Inc. The rat anti-mouse CD200R used was characterized elsewhere[2].

Preparation of cells: Single cell suspensions from different tissues were prepared aseptically by incubation of teased tissue in collagenase for 30 minutes at 37°C, and after centrifugation, cells were resuspended in α-Minimal Essential Medium supplemented with 2-mercaptoethanol and 10% fetal calf serum (αF10). LPS splenic DC, stained (>90%) with DEC205, were obtained by overnight culture (1 μg/ml LPS) of adherent fresh spleen cells.

Cytotoxicity and cytokine assays: In allogeneic mouse mixed leukocyte cultures (MLC), used to assess cytokine production, F13 responder cells were stimulated with equal numbers of mitomycin-C treated (45 min at 37°C) BALB/c spleen stimulator cells in triplicate in αF10. Where cytokine production or proliferation of cells from collagen-immunized mice was measured, cells were stimulated in the presence of 20 μg/ml bovine collagen. For both sets of cultures, supernatants were pooled at 40 hours from replicate wells and assayed in triplicate in ELISA assays for lymphokine production, with capture and biotinylated detection mAbs as described above. Varying volumes of supernatant were bound in triplicate at 4°C to plates pre-coated with 100 ng/ml mAb, washed 3x, and biotinylated detection antibody added. After washing, plates were incubated with strepavidin-horse-radish peroxidase, developed with appropriate substrate and OD_{405} determined using an ELISA plate reader. All assays showed sensitivity in the range 40 to 4000 pg/ml.

Where cytotoxicity was assayed, cells were harvested from MLR cultures at 5 days and titrated at different effector: target ratios for killing (4 hours at 37°C) of ^{51}Cr-labeled P815 tumor target cells.

RESULTS

Suppression of induction of CTL and type-1 cytokines from spleen cells of transgenic mice stimulated with alloantigen in the presence of doxycycline: PBL samples from $F1_3$ mice of ($rtTA^{tg}$ x TRE-CD200-GFP^{tg}) matings were typed for expression of the independent transgenes using the primer pairs described. Each tg mouse parent was at the 3rd generation

of backcross to C57BL/6; $F1_3$ mice derived from four founder lines of TRE-CD200-GFP were used. Spleen cell preparations were prepared from individual, doubly transgenic mice and singly transgenic mice, and cultured in the presence or absence of doxycycline with mitomycin-c treated BALB/c spleen stimulator cells. Cytokines were assayed in supernatants at 40 hours by ELISA, and CTL directed to P815 tumor targets at day 5 of culture. Data, in Figures 3 and 4, show CTL responses and cytokine profiles respectively under these conditions.

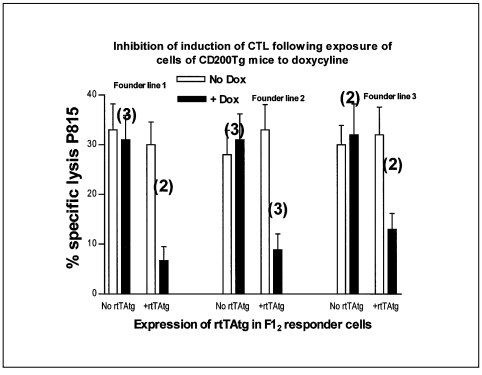

Figure 3. *Inhibition of induction of CTL following allostimulation of spleen cells from doubly transgenic (rtTA^{tg} and TRE-CD200-GFP^{tg}) $F1_3$ mice in the presence of doxycycline. MLR cultures were initiated using BALB/c spleen stimulator cells, and individual responder spleen cells from three different founder lines, after typing PBL from those spleen donors for expression of TRE-CD200-GFP in the simultaneous presence/absence of the rtTA^{tg}. The number of donors used from each founder line is shown in parentheses. CTL assays were performed using ^{51}Cr P815 target cells at day 5 of culture. Data show arithmetic mean (±SD) specific lysis for the different groups at an effector: target ratio of 100:1.*

These data indicate that while exposure to doxycycline *per se* has no significant effect on induction of CTL of IFNγ production in cells obtained from singly transgenic TRE-CD200-GFP mice (no rtTA^{tg}, therefore no induction of CD200 by doxycycline, see Figure 2). In contrast, doxycycline caused profound alterations in MLR responses of cells from doubly transgenic mice. In this case there was inhibition of CTL induction (Figure 3) and decreased IFNγ production, with increased IL-4 (see Figure 4). Similar suppression of CTL and type-1 cytokine production was observed in earlier reports following infusion of a solubilized form of CD200 (CD200Fc)[4], or following *in vivo* induction of CD200 expression in mice following portal vein alloimmunization[5].

Future plans involve further *in vitro* characterization of immune responses (and tissue distribution) of CD200 in these lines, and the effect of CD200 expression on organ survival using these tg/tg mice as donors.

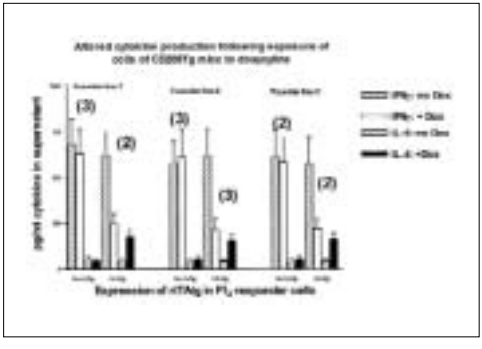

Figure 4. *Altered induction of cytokine production (increased IL-4, decreased IFNγ) measured by ELISA in 40-hour cultures of cells from mice shown in Figure 3. Data show arithmetic mean (±SD) cytokine concentration (pg/ml) for the different groups. Qualitatively equivalent patterns (to IFNγ, IL-4) were seen for IL-2 and IL-10 respectively (data not shown for clarity).*

Construction of CD200R KO mouse, and characterization of its use in transplantation: We have shown that CD200R$^+$ cells were stimulated to produce immunosuppression in the presence of CD200Fc[6], and that in a transplant model, a cross-linking anti-CD200R

antibody was itself immunosuppressive[7]. Hoek and colleagues have also shown that in mice with deletion of the CD200 gene, increased proliferation of CD200R$^+$ cells is observed[1], and that these animals show an increased susceptibility to collagen-induced arthritis and experimental allergic encephalomyelitis (EAE). Together these data support the notion that CD200:CD200R interactions are important in the immunoregulation which contributes to determining susceptibility to transplant rejection and autoimmune disease[1].

CD200 is a relatively ubiquitously expressed molecule, its presence is documented on the surface of, amongst others, follicular dendritic cells, brain, thymus, uterine tissue, activated B cells and endothelial cells. In contrast, it seems that CD200R expression is more restricted. Barclay *et al* reported its presence only on the surface of cells of the myelod lineage, while we have reported evidence also for CD200R expression on the surface of at least a subpopulation of activated αβ and γδ TCR$^+$ cells[6]. There is no data yet available which addresses the issue of whether the CD200Rs expressed on different cell types represent different isoforms of a family of molecules, nor indeed whether there exists functional, as well as perhaps structural, heterogeneity in the CD200R family. Previous work from both this laboratory (unpublished) and that of Barclay *et al*[8] defined the cDNA sequence and mAbs to a CD200R expressed on the surface of macrophages, and at least a subpopulation of activated T lymphocytes[6,8].

In order to investigate the possibility of heterogeneity in CD200R itself, we initially explored evidence for the existence in the genomic database of other CD200R genes. This identified a family of CD200Rs (see Figure 5). Following the genomic identification and mapping of a number of putative CD200R genes we have produced heterologous (rabbit) and rat mAbs to the different isoforms, by synthesis of peptides conforming to unique sequences in the CD200Rs, and immunizing animals with KLH-peptides. We have defined unique tissue and cell distribution patterns for the CD200R isoforms by northern and Southern blotting, and using the anti-CD200Rs, by western gels and FACS analysis, following expression of cDNAs encoding the different CD200Rs in COS cells. We have now begun studies to characterize functional heterogeneity in the CD200R isoforms, by examining the effect of anti-CD200Rs on alloimmune responses (induction of CTL/cytokines) generated *in vitro*. All of these studies are a necessary prelude to the goal of constructing a CD200R KO (we will produce a CD200R2 isoform KO).

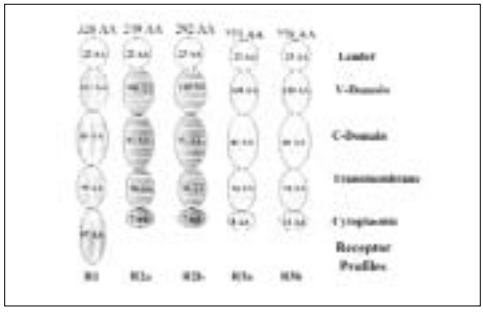

Figure 5. *Tissue distribution of different CD200R isoforms, as defined to date by Southern gels or PCR, and Western blots.*

Evidence for binding of CD200Fc to CD200R isoforms: The various CD200R isoforms identified by genomic mapping, whose cDNAs were ex-pressed in COS cells, were assessed

for their ability to serve as counter-ligands for CD200. An immunoadhesin (CD200Fc) incorporating the extracellular domain of CD200 linked to a murine IgG2a Fc region was used, as described[4]. CD200Fc was coupled to FITC and then examined by FACS analysis binding of FITC-labeled CD200Fc to COS cells, transiently transfected with pBK vectors encoding cDNAs for the various CD200R isoforms. As a control we examined binding to COS cells transfected with empty pBK vectors. COS cells expressing the different CD200R isoforms all bound FITC-CD200Fc.

Anti-CD200R antibodies and CD200Fc immunoadhesin: Based on the predicted amino acid sequence of the CD200R isoforms, the following peptides were synthesized, coupled to keyhole limpet hemocyanin, and used to immunize rabbits or rats for ultimate production of heterologous or rat mAbs to mouse CD200R, essentially as described in a previous report[7].

CD200R1	STPDHSPELQISAVTLQHEGTYTC
CD200R2a	CEAMAGKPAAQISWTPDGD
CD200R2b	KPRGQPSCIMAYKVETKET
CD200R3a	CSVKGREEIPPDDSFPFSDDN
CD200R3b	LQQISKKICTERGTTRVPAHHQSS

Rat mAbs were pre-screened in ELISA using plates coated with the relevant peptides (100 ng/ml). The specificity of all sera and mAbs was assessed by FACS staining, using COS cells transiently transfected with an "empty" pBK vector, or a vector containing a cDNA insert for the respective CD200Rs, followed by FITC-anti-rabbit Ig (or anti-rat Ig). Control cells were stained with FITC antibodies only.

Figure 6 shows the composite staining patterns for all of these tissues with the different sets of mAbs.

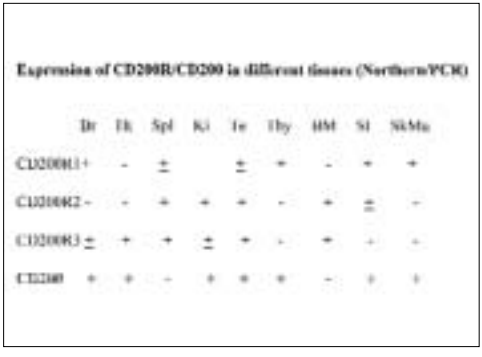

Figure 6: *Schematic representation of structure of different CD200R isoforms.*

TIME-LINE for completion of studies: Our studies to date are progressing well. CD200tg/tg mice are currently breeding to the 4th generation backcrossed onto C57BL/6, and preliminary, *in vitro*, data confirm that they have the predicted phenotype. We anticipate beginning analysis of organ transplants using these as recipients or donors. Analysis of CD200R has proved to be more complicated than anticipated, with evidence for multiple CD200R isoforms with different tissue distribution. This in turn may explain some of the complex properties of CD200:CD200R interactions that have been uncovered. We have chosen to produce a CD200R KO, because of the tissue distribution of CD200R2, and preliminary evidence of the effect

of anti-CD200R2 mAbs on immune reactivity *in vitro* (unpublished). We will have more to present on this work in our progress update in 2003.

References

1. Hoek RM, Ruuls SR, Murphy CA, Wright GJ, Goddard R, Zurawski SM, Blom B, Homola ME, Streit WJ, Brown MH, Barclay AN, Sedgwick JD. *Science* 2000; 290:1768-1771.
2. Ragheb R, Abrahams S, Beecroft R, Hu J, Ni J, Ramakrishna V, Yu G, Gorczynski RM. *Immunol Lett* 1999; 68:311-315.
3. Gorczynski RM, Bransom J, Cattral M, Huang X, Lei J, Xiaorong L, Min WP, Wan Y, Gauldie J. *Clin Immunol* 2000; 95:182-189.
4. Gorczynski RM, Cattral MS, Chen ZG, Hu JA, Lei J, Min WP, Yu G, Ni J. *J Immunol* 1999; 163:1654-1660.
5. Gorczynski RM, Chen Z, Fu XM, Zeng H. *Transplantation* 1998; 65:1106-1114.
6. Gorczynski RM, Yu K, Clark D. *J Immunol* 2000; 165:4854-4860.
7. Gorczynski RM. *Eur J Immunol* 2001; 31:2331-2337.
8. Wright GJ, Puklavec MJ, Hoek RM, Sedgewick JD, Brown MH, Barclay AN. *Immunity* 2000; 13:233-242.

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LAT, a Molecule Critical for T Cell Activation and Function, is a Potential Substrate for Calcineurin

Cyclosporine A (CsA) and FK506 have been extremely useful for treating GVHD, autoimmune and inflammatory diseases and have truly revolutionized allograft transplantation. However, CsA and FK506 have been shown to induce side effects and toxicity. The side effects are due, at least in part, to the fact that calcineurin is found in all cells; thus, blocking calcineurin activation by CsA and FK506 also disrupts the function of cells other than T cells, leading to toxicity. Furthermore, blocking calcineurin appears to affect the function of several proteins in T cells, which may modulate T cell processes in addition to blocking IL-2 production. For example, recent studies have shown that calcineurin, in addition to regulating the function of NFAT, a transcription factor important for IL-2 gene transcription, also regulates the phosphorylation of the transcription factor Elk-1, the IP_3 receptor, and the proapoptotic molecule Bad. Given that blocking calcineurin signaling pathways has proven useful for blocking T cell activation, we initiated studies to define T cell-specific targets for calcineurin that are important for T cell activation. The identification of these targets should be useful for designing drugs that block the activation of such targets and in turn specifically block T cell function with no or minimal side effects.

Studies from our laboratory showed that CsA and FK506 substantially potentiate TCR-mediated increases in the levels of the critical signaling molecule LAT, thereby implicating calcineurin in regulating LAT expression. Further analysis of the effects of CsA and FK506 on T cells revealed that ligating TCR in the presence of FK506 or CsA-induced post-translational modifications in LAT that modulated the migration of the molecule in SDS-PAGE. The shift in the electrophoretic mobility of LAT occurred rapidly (within 5-15 min) after stimulating TCR in the presence of FK506 or CsA, and therefore preceded the increase in LAT expression. Thus, CsA and FK506 regulate not only the expression of LAT, but also its posttranslational modification. CsA and FK506 did not have a detectable effect on the electrophoretic mobility of PLCγ, Pyk2, Vav, Zap, or Grb2, indicating that FK506 and CsA do not lead to a general modulation in the electrophoretic migration of proteins. Notably, calcineurin, which is a major target for CsA and FK506, dephosphorylated LAT *in vitro* and reversed the shift in the electrophoretic mobility of the molecule. Furthermore, calcium ionophores that are known to activate calcineurin blocked the effects of CsA and FK506 on the electrophoretic mobility of LAT. Stimulating T cells with the serine/threonine kinase

activator PMA induced a shift in the mobility of LAT, whereas inhibitors of serine/ threonine kinases blocked the modulation in the electrophoretic mobility of LAT. Thus, LAT is a target for serine/threonine kinases and for calcineurin. Furthermore, these results suggest that these enzymes regulate the serine/threonine phosphorylation of LAT, which in turn affects the molecule's electrophoretic mobility.

We embarked on purifying LAT from T cells activated through TCR ligation in the presence or absence of FK506. The studies proved very demanding because a large number (10^{10}) of T cells was needed and because commercially available antibodies appear to have low affinity to LAT. However, after numerous trials, LAT was purified in microgram quantities and was sent for Mass Spectrometry. Once the residues that become phosphorylated upon TCR ligation in the presence of FK506 have been identified, we will determine the effect of targeting those residues on the following:

1) LAT function, including its tyrosine phosphorylation, its association with signaling molecules, its palmitoylation, and its localization to the glycolipid-enriched membrane microdomains (GEM).

2) TCR-mediated signaling, including protein tyrosine phosphorylation, Ca^{2+} influx, and the activation of mitogen-activated protein kinase (MAPK) cascades.

3) TCR function, including the expression and production of cytokines and the initiation of activation-induced cell death.

4) The responses of TCR-stimulated T cells to FK506 and CsA, including the production of cytokines such as IL-2, TGFβ and IFNγ.

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The Role of TRANCE/RANK Interaction during Allogenic Immune Responses

The major goal of this study is to understand the role of a recently described member of the tumor necrosis factor (TNF) superfamily called TRANCE (TNF-related activation induced cytokine) and its receptor RANK (receptor activating NF-κB) during allogeneic immune responses *in vivo*. In the immune system, TRANCE is expressed by activated T cells and RANK is mostly expressed by mature dendritic cells (DC) that are important antigen-presenting cells. TRANCE induces survival and activation of mature DC. Despite the critical role of the CD40L pathway of co-stimulation in antigen presenting cell functions, the TRANCE pathway can mediate CD40L-independent T cell activation. Our hypothesis is that TRANCE is involved in DC-mediated T-cell priming during allograft rejection. In preliminary experiments supported by the ROTRF grant, we have shown that TRANCE mRNA is strongly upregulated in acutely rejected heart allografts in rats and that the inhibition of the TRANCE-RANK pathway led to enhanced allograft survival.

Aim 1. *To extend our study on the regulation of expression of TRANCE and RANK during acute and chronic rejection.* In preliminary experiments we have studied the expression of TRANCE and RANK mRNA in acutely rejected heart allografts in rats. We found that both molecules were strongly upregulated during acute rejection. To study the expression of TRANCE and RANK protein in the same acute rejection model and in models of chronic allograft rejection we decided to generate monoclonal antibodies against rat TRANCE and RANK. We also intend to generate blocking monoclonal antibodies that will be useful for *in vivo* blocking experiments. We cloned rat TRANCE and rat RANK by PCR, using primers specific for conserved regions between mouse and human mRNA sequences. A fusion molecule encompassing the ectodomain of rat TRANCE fused to the ectodomain of the human CD8 molecule has been constructed and is currently being used to immunize Balb/c mice. We are generating stable transfectants expressing the ectodomain of rat TRANCE that will be used to screen hybridoma supernatants. A similar approach has been used to generate monoclonal antibodies to rat RANK. A rat RANK-hIgG.Fc portion fusion molecule has been generated and will also be used to immunize mice. These monoclonal antibodies will be used *in vitro* and *in vivo* during the last year of our ROTRF funding.

Aim 2. *To analyze immunological mechanisms of allograft enhancement by the RANK.Fc fusion molecule.* Our preliminary experiments have shown that TRANCE blockade using the mRANK.Fc fusion molecule induced prolongation of heart allograft survival in rats (Table I). To analyze the effect of prolonged TRANCE blockade we have generated a recombinant adenovirus coding for RANK.Fc. Using *in vitro* control experiments, we have shown that transduced COS cells expressed the recombinant protein (immunohistology) and produced RANK.Fc in their supernatants (ELISA). Hearts from LEW.1W donors were transduced with recombinant adenoviruses by slow injection into the apex and ventricular walls at four different points. A strong expression of RANK.Fc was observed in transduced allografts on day +5 and high levels of RANK.Fc were detected in the sera of these animals one month after grafting. Similar to allograft recipients injected with RANK.Fc protein, rats that received an allograft transduced with RANK.Fc adenovirus exhibited a prolonged graft survival as compared to control Addl324 adenovirus (Table I). These results indicate that prolonged TRANCE blockade does not appear to improve allograft survival further than that achieved by short-term blockade.

Table 1. *Allograft survivals*

Groups	Survival (days)	Survival (MST ± SD)	*p* value
Untreated	5, 5, 6, 6, 6, 7, 7, 7, 9,10	6.8±1.6	
0.5 mg human IgG on days 0, +2, +4, IP	7, 7, 7, 7	7±0	NS (vs untreated)
0.5 mg TR.Fc on days 0, +2, +4, IP	10, 11, 20, 45	21.5±16	0.01 (vs hIgG)
1 mg TR.Fc day 0, IV	8, 8, 9, 34	14.8±12.8	0.0003 NS (vs hIgG)
1 mg TR.Fc on days 0,+2, IV	14,21,25,44	26±12.8	0.0003 (vs hIgG)
Addl324-transduced allografts	6,6,7,7,7,7,7,8,10,11	7.6±1.6	
Ad.RANK.Fc-transduced allografts	10, 11, 11, 12, 13, 14, 15, 16, 16,18, 21	14.3±3.3	<0.0001 (vs Addl324)

We investigated the mechanisms of allograft enhancement by TRANCE blockade in recipients of Ad.RANK.Fc-transduced allografts. RANK.Fc transduced allografts exhibited a strong leukocyte infiltrate similar to that observed in control allografts. Immunostaining revealed a substantial decrease in both the numbers and intensity of staining of CD11b positive cells. Other markers were not modified (MHC class II, CD4, CD8, CD25). TRANCE blockade did not inhibit anti-donor alloantibody production. We did not observe any decrease in the expression of IFN-γ, IL-2, IL-4, IL-10, TNF-α or TGF-β mRNA in RANK-Fc transduced allografts as compared to controls on day 5 after grafting. However, the expression of CD40L mRNA was strongly increased in RANK-Fc transduced allografts. This suggests that TRANCE blockade might upregulate CD40L on activated T cells that could compensate for the lack of TRANCE/RANK signaling in mature dendritic cells and therefore eventually lead to acute rejection. This hypothesis will be tested in a simplified *in vitro* system during the last year of our ROTRF funding.

Aim 3. *To optimize the effect of TRANCE blockade on acute and chronic allograft rejection.*
We have focused on the effect of combined TRANCE and CD40L blockade on chronic heart allograft rejection. These experiments are in progress.

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Identification of Novel Proteins Involved in Rejection of Transplanted Organs

The principal goal of our application was to identify a role for two key transcription factors (STAT5a/b) involved in mediating T cell activity and allograft rejection. Within this proposal we suggested three approaches to inactivate these proteins and ultimately determine their effects *in vitro* and *in vivo* using T cell cultures and rat heart allograft transplant models, respectively. At the conclusion of the second year of support, we have made significant strides towards successfully attaining the major aims of this proposal.

SPECIFIC AIM 1. Selective inhibition of Janus tyrosine kinase (JAK) 3 blocks STAT5a/b activation and T lymphocyte function. We have satisfied all of the sub-aims within Specific Aim 1 by identifying two selective inhibitors (AG-490 or PNU156804) that block STAT5a/b tyrosine and serine phosphorylation. Both agents disrupted Stat5a/b activity following stimulation of T cells with various T cell growth factors, including interleukin (IL)-2 and other related cytokine family members (IL-7, IL-9 and IL-15). We have identified PNU156804 as a selective Janus tyrosine kinase (JAK) 3 inhibitor, in contrast to JAK2, in blocking the STAT5 signalling pathway. As such, we found that inhibition of STAT5a/b phosphorylation blocked their ability to translocate to the nucleus and bind DNA, thus ablating their ability to regulate T cell-mediated gene transcription. We confirmed this *in vivo* using a Lewis to ACI rat heart transplant model. STAT5a/b DNA-binding activity was blocked in lymphocytes isolated from treated recipients compared with control animals. We also demonstrated that inhibition of this signalling pathway *in vivo* significantly prolonged allograft survival, and that it can act synergistically when the signal 1 pathway is inhibited with sub-therapeutic doses of cyclosporine A (CsA), but not another signal 3 inhibitor, rapamycin, which displayed weakly additive effects. Lastly, we found that inhibition of the STAT5a/b pathway reduces mononuclear cell infiltration and damage of heart allografts in transplanted recipients.

SPECIFIC AIM 2 (YEAR 2). Measure immunosuppressive potential of dominant negative forms of STAT5a/b using non-viral gene delivery As a second approach to disrupting STAT5a/b activity, we have generated dominant negative constructs of STAT5a/b to inhibit T cell activity. Due to low transfection efficiency for this Stat5a/b gene product using our adenoviral and retroviral delivery systems, successfully infected T cell lines are being selected, cloned and assayed for the effects of this altered protein on T cell activity.

SPECIFIC AIM 3 (YEAR 3). Selective inhibition of STAT5a/b by antisense oligonucleotides blocks T cell function and rejection of transplanted organs. No work has been published on the use or design of antisense oligonucleotides for STAT5 protein suppression. We have accomplished two major steps in this aim by successfully designing and delivering novel STAT5 antisense-oligonucleotides with high efficiency in primary T cells and T cell lines. These antisense oligonucleotides optimally suppress STAT5a/b protein expression in lymphocytes, but fail to affect the expression of closely related proteins including STAT3. Scrambled oligonucleotides of the same chemistry had little effect on these cells.

Extensive preliminary data with this reagent now suggest that STAT5a/b provide a critical role as T cell survival factors based on annexin V, propidium iodide and TUNNEL assays. This contradicts data generated from STAT5a/b gene-deficient mice. Ongoing studies are addressing these differences. However, our data suggest that abbrogation of both STAT5a/b protein levels (within 12 hours) by antisense oligonucleotides can lead to apoptosis of IL2-responsive primary and tumor T cell lines within 24-48 hours. Putative apoptotic cell signalling pathways mediating this event are being investigated to explain these observations. Compelling evidence also support the notion that co-culture of antisense-targeted cells can be rescued with high concentrations of IL-2, suggesting that the recruitment of alternative, non-STAT5a/b signalling pathways, can protect these cells. Future studies are planned to test the efficacy of these antisense *in vivo* to disrupt T cell activity and their possible therapeutic role in ablating allograft rejection using a heterotopic rat heart transplant model.

Publications

1. Stepkowski SM, Erwin-Cohe RA, Behbod F, Wang M-E, Qu X, Tejpal N, Nagy ZS, Kahan BD, Kirken RA. A selective inhibitor of Janus tyrosine kinase (JAK) 3, PNU156804, prolongs allograft survival and acts synergistically with cyclosporine but additively with rapamycin. *Blood* 2002; 99:600-609.
2. Kirken RA, Stepkowski SM. New directions in T cell signal transduction and transplantation tolerance. *Curr Opin Organ Transplant* 2002; 7:18-25.
3. Stepkowski SM, Nagy ZS, Kahan BD, Kirken RA. The role of signals1, 2 and 3 in induction of transplantation tolerance. *Transplant Proc* 2001; 33:3831-3833.
4. Stepkowski SM, Kirken RA, Nagy ZS, Trawick BW, Wang M, Tejpal N, Wang M, Tian L, Clark J, Kahan BD. The role of Stat5 in the induction of regulatory T cells in transplantation tolerance. *Transplant Proc* 2001; 33:3835-3836.
5. Kirken RA. Targeting JAK3 for immune suppression and allograft acceptance. *Transplant Proc* 2001; 33:3268-3270.

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Can ES Cells Form a Specific Complex Organ?

We will report two research efforts. The first is a direct continuation of our original proposal and describes the effect of ES cells on outgrowths observed in mutant mouse embryos that lack *Fgfr2b* function and display no limb outgrowth. The second effort is based on a new finding from our laboratory. These results show that the *Fgfr2c* alternative is a positive regulator of bone development and its gain of function mutation activates numerous aspects of bone formation. We wish to utilize this observation for an ES cell therapy of osteoporotic or osteoarthritic bone loss. The idea is to prepare ES cells carrying homozygous gain of function mutations of *Fgfr2c* to influence the healing of bone fractures.

Part 1

The recessive *Fgfr2* mutants used in these experiments are defective in limb and branching morphogenesis and die at mid-gestation[1-3]. Our mutation affects the entire *Fgfr2* locus. Since the specific *Fgfr2b* limb phenotype arises earlier than the viable craniosynostosis and dwarfism due to loss of *Fgfr2c,* this program analyzes a defect connected to the IIIb alternative. As described in the original proposal, we have studied cultures of trunk fragments of these limb-less embryos. The culture system employed allows embryo fragments at the pre-limb outgrowth stage (9.5-10.0 d.p.c) to develop limb buds with distinguishable falangeal rays in four days in culture[5]. We injected normal ES cells into the trunk fragments and studied their growth, histological architecture, the origin of the cells in the outgrowth, and the genes expressed during this process.

We have established in this project that most injected embryos display a more or less amorphous outgrowth at the injected side. The outgrowth consists of chondroblasts, chondrocytes and primitive mesenchymal elements. Introduction of a β-galactosidase cassette into the mutant ES cell clone allowed the origin of the induced outgrowth to be traked. Interestingly, both recipient-type mutant and donor-type wild-type chondrocytes could be found. This suggests that the graft differentiating towards the chondrocyte lineage induces mutant stem cells, which normally would form no limb cartilage, to follow suit. This finding suggests that the mutant cells are capable of differentiating into chondroblasts and chondrocytes provided that wild-type derived environmental factors are present. This important observation requires further investigation.

Teratomas formed by subcutaneously injected ES cells frequently form cartilage, although this usually takes several days. It is therefore crucial to know whether cartilages that differentiate

in the presence of wild-type ES cells follow the structure of the limb skeleton. Although some preparations look encouraging, improved whole mount and histological evidence and a statistical evaluation of this problem are needed before a decisive conclusion can be reached.

It was essential to know at this stage whether the ES cell-induced growths express genes characteristic of limb development. The most important genes are genes, such as Fgf8, that are normally expressed in the apical ectodermal ridge (AER), an epithelial growth center of the limb bud. The AER is responsible for proximal-distal limb outgrowth. As Figure 1 demonstrates, untreated embryo fragments of mutant origin do not express FGF8, as shown by whole mount *in situ* hybridization in the left side of the figure. After injecting wild-type ES cells into mutant embryos, the cultured fragments expressed FGF8 within two days of incubation (right side of Figure 1). Closer inspection reveals two ridge-like areas of strong FGF8 expression of limb outgrowth (left side of Figure 1). Additional experiments will be carried out to clarify whether the FGF8 label is associated with normal AER structure. At face value this result is an important advance towards validating our hypothesis. It suggests that the outgrowth observed includes genuine characteristics of limb development.

To sum up, two main observations were made in this project period: wild-type ES cells induce cartilage proliferation in *Fgfr2*$^{-/-}$ embryo fragments; and this process is associated with the expression of bona fide AER genes, such as FGF8.

Difficulties of the project: A major recurrent difficulty has been the sensitivity of the culture system. In most experiments, considerable development was observed when wild-type and not when mutant ES cells were injected into mutant embryo fragments (Table 1). Nevertheless, there were periods when no development was observed. Modification of the culture medium may overcome this problem.

Table 1. *R2, mutant ES cells transplanted into the normal forelimb, create extra growth compared with ES mutant cells*

#	R2$^{-/-}$ES	Remarks	#	WT ES cells	Remarks
1	+		11	++	Deformed
2	++		12	+++	Deformed
3	+/-		13	+++	Extra growth
4	+		14	++	
5	++		15	+++	
6	+		16	+++	Extra growth
7	+/-	Extra growth on one side	17	+++	Extra growth + digits
8	+		18	++	Extra growth
9	++		19	+++	
10	+/-		20	+++	Extra growth on one side

Part 2

Use of mutant ES cells expressing a positive regulator of bone growth for the treatment of bone fractures. The planned stem cell therapy experiment is based on results obtained in a separate project, which is now under evaluation. In this genetic study we investigated the function of the IIIc transcriptional alternative of *Fgfr2*. This alternative is expressed in mesenchymal cells and during later development in the skeleton[6,7]. Mutations of FGFR2c that create intra-chain disulfide bonds or other changes that stabilize receptor dimerization are associated with craniosynostosis and limb defects (fused fingers and/or toes) in man[8]. We created two *Fgfr2c* mutations. In the first, a stop codon and a new restriction enzyme site was created in exon 9, which inactivated this receptor variant, without affecting the IIIb variant in the *Fgfr2b* variant[4]. This mutation caused a loss-of-function phenotype, which manifested as recessive viable dwarfism with skull base craniosynostosis and late ossification of the appendicular and axial skeleton.

Significantly, this mutation resulted in downregulation of *Spp1, Cbfa1, Ihh* and *PTHrP,* which are bone development genes in osteocyte and chondrocyte lineages. In the second "knockin" mutant, the disulfide-bond-forming cysteine of exon 9 was mutated to glutamine. This change, typical of Crouzon-type craniosynostosis in man, causes ligand-independent activation of the FGFR2c receptor; hence it is a gain-of-function mutation. Heterozxygous mice are viable, with skull vault craniosynostosis and up-regulated bone development genes. The homozygote, which is unknown in man, is perinatal lethal with cleft palate, agenesis of the knee, carpal and tarsal joints, connected with strong upregulation of bone development genes.

We interpret these results to suggest that FGFR2c is a positive regulator of bone development. Its effect is opposite to that of another FGFR isotype, FGFR3, which inhibits chondrocyte and osteoblast development[9,10]. Since both genes are epistatic to most bone development genes, it is reasonable to assume that they affect osteogenesis from its early stages.

Based on these findings we would like to develop a stem-cell therapy approach. We assume that ES cells homozygous for the gain-of-function allele of *Fgfr2c* should support fracture healing. Preparation of ES cell clones homozygous for the mutation and a simple bone-fracture-healing protocol could be the first test of this hypothesis. Defective bone reorganization is a major clinical problem. Outstanding examples are incomplete healing of osteoporotic fractures and the bone and cartilage loss observed in osteoarthritis. These significant diseases have no definitive, hypothesis-driven therapy, for the time being.

References

1. Arman E et al. Fgfr2 is required for limb outgrowth and lung branching morphogenesis. *Proc Natl Acad Sci USA* 1999; 96:11895-11899.
2. De Moerlooze L et al. An important role for the IIIb isoform of fibroblast growth factor receptor 2 (FGFR2) in mesenchymal-epithelial signalling during mouse organogenesis. *Development* 2000; 127:483-492.
3. Revest JM et al. Fibroblast growth factor receptor 2-IIIb acts upstream of Shh and Fgf4 and is required for limb bud maintenance but not for the induction of Fgf8, Fgf10, Msx1, or Bmp4. *Dev Biol* 2001; 231:47-62.

4. Eswarakumar VP et al. The IIIc alternative of Fgfr2 is a positive regulator of bone formation. *Development* 2002; in press.
5. Ochiya T et al. Hst-1 (FGF4) antisense oligonucleotides block murine limb development. *J Cell Biol* 1995; 130:997-1003.
6. Orr-Urtreger A et al. Developmental localization of the splicing alternatives of fibroblast growth factor receptor-2 (FGFR2). *Dev Biol* 1993; 158:475-486.
7. Peters K et al. Unique expression pattern of the FGF receptor 3 gene during mouse organogenesis. *Dev Biol* 1993; 155:423-430.
8. Wilkie AO. Craniosynostosis: genes and mechanisms. *Hum Mol Gen* 1997; 6:1647-1656.
9. Deng C et al. Fibroblast growth factor receptor-3 is a negative regulator of bone growth. *Cell* 1996; 84:911-921.
10. Colvin JS et al. Skeletal overgrowth and deafness in mice lacking fibroblast growth factor receptor 3. *Nat Genet* 1996; 12:390-397.

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Effect of T Cell Costimulatory Blockade and Bone Marrow Transplantation on Organ Transplant Rejection

It has long been recognized that different allogeneic organs and tissues elicit recipient immune responses of different strength and character. Both experimental and clinical evidence demonstrate that intestinal grafts are amongst the most immunogenic of all transplanted organs. Using a murine model of intestinal transplantation, we have demonstrated that either $CD4^+$ or $CD8^+$ T cells are sufficient to mediate the destruction of transplanted intestines. Furthermore, many therapeutic regimens that promote the long-term survival of other transplanted organs fail to prolong the survival of intestinal allografts as a consequence of their inability to inhibit rejection mediated by $CD8^+$ T cells. Our studies demonstrate that depletion of $CD4^+$ T cells, blockade of the LFA-1/ICAM-1 pathway, and blockade of the CD28/B7 and/or CD154/CD40 pathways fail to substantially prolong the survival of intestinal allografts. We therefore sought to identify alternative approaches that promoted the long-term survival of intestinal allografts.

The advent of non-myeloablative strategies that result in hematopoietic chimerism following bone-marrow transplantation suggested a clinically applicable approach to induce tolerance following the transplantation of highly immunogenic organs. In order to test this hypothesis, C57BL/6 mice were treated with the following regimen:

- Blockade of the CD28/B7 pathway using CTLA4-Ig (500 mg administered days 0, 2, 4, and 6)
- Blockade of the CD154/CD40 pathway using an anti-CD154 mAb (500 mg) administered on the same days
- Depletion of early hematopoietic stem cells using busulfan (600 mg) administered on day 5
- Infusion of 20 x 10^6 BALB/c bone marrow cells on days 0 and 6
- Transplantation of a segment of small bowel from BALB/c donors on day 10 to 14

Chimerism and depletion of donor-reactive cells were assessed by flow cytometry. Allografts were histologicaly assessed for immunologic damage at predetermined time points. Grafts were scored as follows 0, normal; 1, mild acute rejection; 2, moderate rejection; and 3, severe rejection.

RESULTS. Donor-strain cells of hematopoietic origin (granulocytes, macrophages, B and T cells) were first detected 20 to 30 days after the second infusion of bone marrow cells. Depending upon the lineage examined, the degree of chimerism continued to increase until day 50 to 75. Mice were followed for up to 100 days following intestinal transplantation at which time the mean percentage of donor-derived hematopoietic cells ranged from 25 to 55%. As shown in Figure 1, this regimen resulted in long-term survival of intestinal allografts. It is important to note that all long-term surviving allografts displayed normal histology and lacked any evidence of chronic rejection.

Two allografts examined at an earlier time point (day 50) displayed mild acute cellular rejection. This observation suggests that some recipients may develop an immune response to the intestinal allograft that is self-limited. Figure 1 also illustrates that combined co-stimulation blockade and bone-marrow infusion without busulfan had no beneficial effect on allograft survival. Utilizing reactivity to endogenous superantigens as a surrogate for alloreactivity, we demonstrated that chimeric mice had significantly decreased numbers of superantigen-reactive T cells (those with a TCR comprised of either Vβ5 or Vβ11) but normal numbers of T cells that utilized a Vβ chain that did not recognize the superantigen in the context of I-E (i.e., Vβ8). These data demonstrate that deletion of donor-reactive cells occurs in chimeric mice and suggest that the long-term survival of intestinal allografts in chimeric recipients may be a consequence of the deletion of donor alloantigen-reactive T cells.

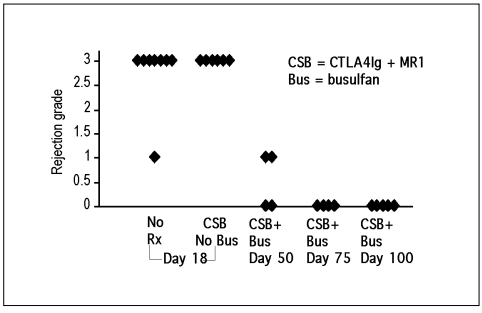

Figure 1. *Inhibition of rejection by bone marrow transplantation.*

Although deletion is likely to be an important mechanism for maintaining the long-term survival of allografts in this model, other mechanisms may contribute to the inhibition of rejection especially at early time points. This is suggested by the observation that significant deletion of donor-reactive cells is first detected at approximately day 30 and is not complete until day 50 to 60, together with the observation that combined costimulation blockade and bone marrow infusion fail to prolong survival (Figure 1). In a number of models the development of a regulatory population of T cells has been associated with long-term allograft survival. In the combined costimulation blockade, busulfan and bone marrow infusion model, the depletion of $CD4^+$ T cells prevents the development of chimerism suggesting that a $CD4^+$ regulatory cell may contribute. However, our observation that chimerism can be induced using this approach in $CD4^{-/-}$ recipients, suggests that if regulation is an important mechanism that contributes to the development of chimerism and long-term allograft survival, the regulatory cells may not be confined to T cells that express CD4. With regard to regulation, two mice developed mild rejection at day 50. We hypothesize that this is a consequence of waning

regulation at a time that precedes complete deletion of donor reactive cells. Future studies will focus on testing this hypothesis and further examining the role of regulatory cells in this model. As an initial approach, in collaboration with Dr. Charles Orosz (Ohio State University), we will use the *trans vivo* DTH assay in an attempt to demonstrate the presence regulatory cells in this model.

A second interesting observation is that although chimeric mice bearing intestinal allografts display donor-specific unresponsiveness *in vitro* (Figure 2), they remain capable of rejecting donor-strain skin allografts *in vivo* (Figure 3). This suggests that although mice treated with combined costimulation blockade, busulfan and donor bone marrow, enjoy long-term survival of intestinal allografts and are unresponsive to donor antigens *in vitro*, they are not tolerant in the strictest sense of the term. Seven of the eight chimeric mice bearing intestinal allografts (BALB/c) that subsequently underwent skin grafting with BALB/c skin, rejected the skin allografts by 37 days. It is important to note that the survival of BALB/c skin grafts (original donor strain) was significantly prolonged relative to third party skin grafts and that despite rejecting the BALB/c skin grafts, histology of the BALB/c intestinal allografts remained normal.

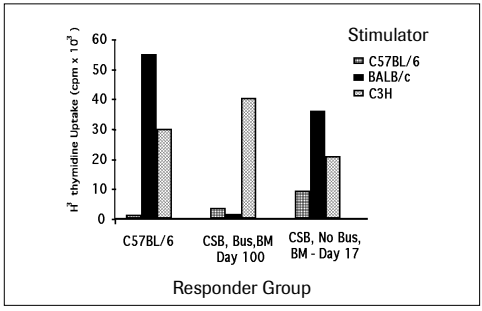

Figure 2. *Donor-specific unresponsiveness in vitro.*

Our data demonstrate that the infusion of donor bone marrow cells using busulfan as a conditioning agent together with a short course of combined blockade of the CD28 and CD154 pathways promotes the long-term survival of highly immunogenic intestinal allografts. The mechanisms responsible for this effect have not yet been fully characterized. Our data suggest that mechanisms other than deletion may play an important role. Additional studies will be necessary to test the hypothesis that regulatory cells contribute to the enhanced allograft survival observed in this model. In addition, it will be important to understand how mice that have deleted at least a subset of donor-reactive cells reject donor-strain skin allografts while maintaining histologically normal intestinal allografts.

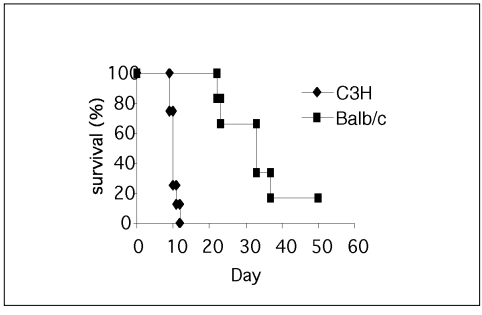

Figure 3. *Donor-specific hyporesponsiveness in vivo.*

Presentations

Long-term survival of intestinal allografts in mice rendered chimeric by bone marrow transplantation with costimulatory blockade. Wang J, Guo Z, Adams AB, Dong Y Durham M, Kim O, Hart J, Pearson TC, Larsen CP, and Newell KA. Presented at the Second Annual Erie Forum for Transplant Immunobiology, Columbus, OH. November 5-6, 2001.

Bone marrow transplantation promotes the long-term survival of intestinal allografts. Wang J, Guo Z, Adams AB, Dong Y, Shirasugi N, Kim O, Hart J, Pearson TC, Larsen CP, and Newell KA. Presented at the American Transplant Congress, Washington, DC. April 26 – May 1, 2002.

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Derivation of Insulin-Producing Cells from Embryonic Stem Cell Lines

A better understanding of islet ontogeny and the phenotype of putative islet stem cells would further improve our ability to generate a cell replacement therapy for treating diabetes. However, it has proven difficult to isolate islet stem/progenitor cells because of their uncertain phenotype and transient existence during development. Because pluripotent embryonic stem (ES) cells are derived from the blastocyst inner cell mass, the embryonic ancestors of all adult tissues, they are a potential source for generating tissue progenitor cells *ex vivo*. Differentiating in culture, ES cells follow many critical developmental stages found in normal embryos, including initial specification of lineage-restricted progenitor cells, ultimately resulting in the formation of highly specialized post-mitotic cell types. Based on these features, ES cells are recognized as a valuable tool for studying commitment and differentiation of mammalian (and now specifically human) embryonic tissues under *in vitro* conditions.

The overall goal of this project is to develop an *in vitro* ES cell-based differentiation system to study pancreatic islet development. In addition, an ES cell-based strategy could permit the generation of an unlimited supply of insulin-producing β cells from an abundant, renewable, and readily accessible source for transplantation.

Previously, we have found that under simple *in vitro* culture conditions without the addition of exogenous growth factors, mouse ES (mES) cells are able to differentiate into cells expressing each of the four major islet hormones, insulin (INS), glucagon (GLU), somatostatin (SOM), and pancreatic polypeptide (PP). Islet precursor cell markers, peptide YY (YY) and islet amyloid polypeptide (IAPP) were expressed abundantly in focal clusters and many, but not all, INS expressing cells. GLU expressing cells were found to co-express both early endocrine markers, indicating the presence of islet precursor cells among mature α and β cell types. Islet restricted transcription factor genes, including *PDX1, neurogenin 3, PAX4, Nkx6.1, and Nkx2.2,* are transcribed by mES cell progeny.

RESULTS

Mouse ES cells differentiate into pancreatic and islet precursor cells. When we immunostained early post-embryoid body (EB) cultures of mES cells with anti-PDX1, we found that many $PDX1^+$ cells were seen, some of which were also PYY^+ (Figure 1). The large number of $PDX1^+$ cells identified in differentiating ES cell cultures contrasts with the relatively uncommon GLU^+, $IAPP^+$, INS^+, SOM^+, or PP^+ cells seen at this stage of development. In mouse cultures,

PDX1 was occasionally co-expressed with INS (probably representing more mature islet β cells. However, many PDX1$^+$ cells did not co-express INS suggesting that these might represent uncommitted pancreatic precursors.

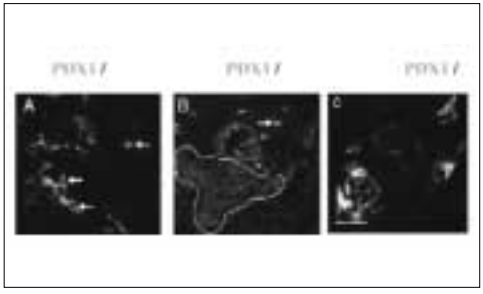
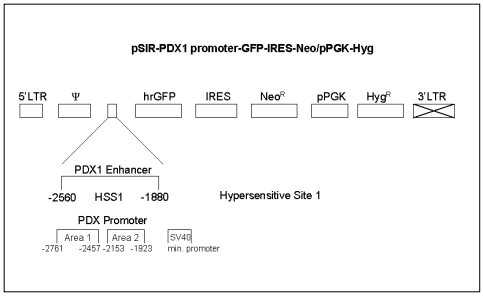


Figure 1. *PDX1 expression in mES cell differentiated cultures. A and B) PDX1 and the early islet marker, YY, are co-expressed in differentiated mEB derivatives 2 and 4 weeks after plating. Nuclear staining of anti-PDX1 was prevalent, frequently exhibiting co-staining with YY (arrows). Large clusters of PDX1$^+$, YY$^-$ cells are also seen (circled region). Some PDX1$^+$ cells co-express INS, probably representing islet β cells. Non–INS expressing PDX1$^+$ cells may represent early pancreatic precursors similar to PDX1$^+$ cells found in the early foregut epithelial bud. (200 X)*

Generation of a lineage selection construct in a self-inactivating retrovirus for transfection of mouse ES cells. Based on genetic selection strategies developed for selecting specific cell types from heterogeneous ES cell cultures, we have designed a double selection transgene construct that will facilitate the selection of PDX1$^+$ expressing pancreatic precursor cells. The construct is shown below.



Enrichment of PDX1$^+$ cells using selective growth factor-supplemented culture conditions. Despite the presence of islet endocrine cells and PDX1+ pancreatic precursor cells among mES cell differentiated progeny, these phenotypes represent only a small minority of the cells within differentiated cultures. In the presence of serum, cultures are prominently heterogeneous in their phenotypes. Therefore, we sought to establish culture conditions that allow the selective expansion of PDX1$^+$ cells. Prior studies have suggested that keratinocyte growth factor (KGF) and nico-

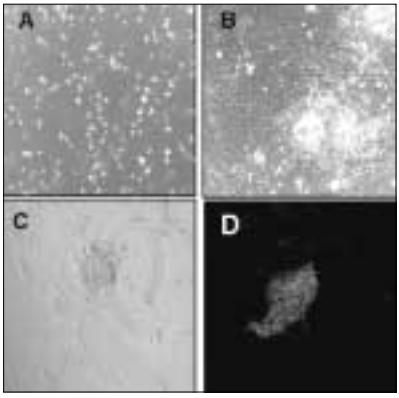


Figure 2. *Selection of PDX1$^+$ colonies from differentiating mES cell cultures. A) Phase contrast of D3 cells differentiated (EB 7 + 21) in the presence of serum and standard culture media (DMEM), and then trypsinized and filtered to generate a single cell suspension and then plated for 24 hours. B) Cells in panel A grown in serum without added growth factors for an additional 10 days. C) Cells in panel A grown under serum-free conditions in the presence of nicotinamide (10 mM), transferrin (10 mg/ml), and selenium (5 ng/ml). D) Colony in C fixed and immunostained with anti-PDX1 (red). The majority of remaining viable cells express PDX1. (200X)*

tinamide (NIC) treatment of pancreatic ductal epithelium are able to enhance the differentiation of islet endocrine cells from this tissue and that culture medium containing insulin, transferrin, and selenium is able to enrich differentiating mES cell cultures for nestin + neural progenitors. Thus, we evaluated these growth factors for promoting growth or enhancing selection of mES cell-derived PDX1$^+$ pancreatic precursors. When D3 cells differentiated in the presence of serum for four weeks are removed from serum and placed in medium containing NIC, transferrin, and selenium (NTS), significant cell death occurs in the culture, yet individual cells survive, proliferate, and ultimately yield colonies that exhibit nearly homogeneous PDX1$^+$ staining throughout the colony. In addition, nearly all such colonies within the culture stain positively (Figure 2). Thus, NTS containing media promotes the selective survival and growth of PDX1$^+$ precursors from mES cell differentiated cultures.

Publications

1. Odorico J, Jacobson L, Hullett D, Thomson J, Kahan B, Differentiation of pancreatic islet endocrine cells from mouse embryonic stem cells in culture. Manuscript in preparation.

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Dr. Daniel N. Streblow, Co-Applicant
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Role of Viral Chemokine Receptors in Cytomegalovirus-Accelerated Transplant Vascular Sclerosis

Transplant vascular sclerosis (TVS), the vascular lesion associated with chronic graft rejection, is characterized by concentric neo-intimal smooth muscle cell accumulation resulting in vessel narrowing and ultimately graft failure. Cytomegalovirus (CMV) accelerates the development of TVS in human and rodent solid organ transplant recipients, and is associated with higher rates of graft rejection, earlier onset, and severity of TVS. The mechanisms behind CMV's link to TVS may involve recruitment of inflammatory cells and smooth muscle cells (SMC) by chemokines. Chemokines are a group of inducible, small molecular weight cytokines that promote cellular migration and activation. These molecules and their receptors play major roles in the development of vascular disease as they are important stimuli for the infiltration of inflammatory cells and the migration/proliferation of SMC. The expression of CC-chemokines, which include MCP-1, MIP-1α, and RANTES, are upregulated in the vascularized graft during ischemia/reperfusion injury, acute rejection, and chronic rejection. Other researchers have demonstrated that the chemokines Fractalkine and IP-10 are also crucial mediators of acute and chronic rejection. By contrast, long-term graft acceptance has been attributed to the absence of chemokine expression, thus substantiating a major role for chemokines in allogeneic graft rejection and during the development of TVS. Importantly, CMW encodes for a CC-chemokine and CC-chemokine receptors and modulates host chemokine expression *in vivo*.

AIMS. The aim of this project is to determine the role of CMV in the acceleration of TVS formation in a rat heart transplantation model by studying the kinetics of TVS, virus presence and CC-chemokine expression, and immune cellular infiltration in the grafts. The aims of the third year of this project will be to determine the specific cell types of the graft vascular lesions that harbor virus, by *in situ* and immunohistochemical techniques. In addition, using a panel of antibodies to different kinetic classes of rat CMV (RCMV)-encoded genes and RCMV-genome arrays, the virus gene expression will be mapped during the process of chronic rejection.

RESULTS. To elucidate the mechanisms involved in CNW-accelerated TVS, first, we determined the kinetics of TVS in graft hearts with or without RCMV infection. For these studies, F344 hearts were transplanted heterotopically into Lewis recipients, treated with low dose CsA, and acutely infected with RCMV. Syngeneic transplant recipients and uninfected

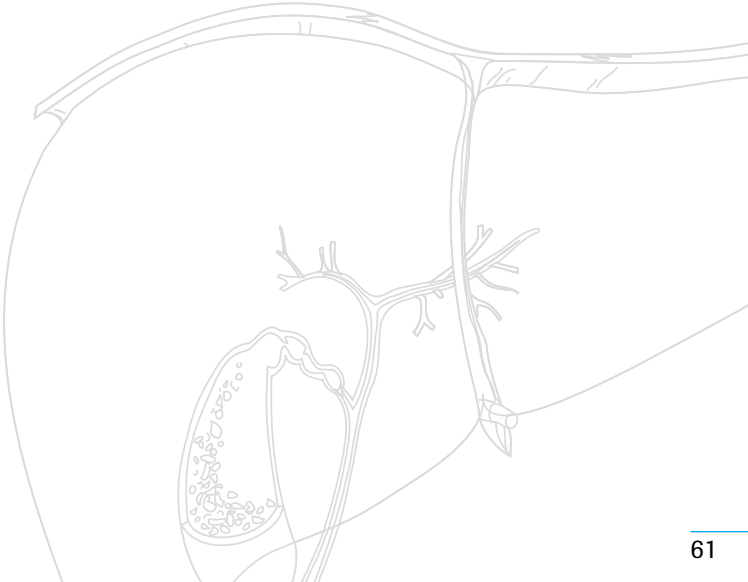
allograft recipients served as controls. Animal grafts and native hearts were harvested at days 7, 14, 21, 35, 45 days (45 days is the mean time to develop CR and TVS in CMV-infected allo-recipients). Within the last year we have studied additional time points at 24, 28, and 32 days post transplantation to further elucidate the sequential events involved in RCMV-accelerated TVS. The transplanted hearts were evaluated histologically, and TVS was assessed morphologically as the mean percentage of vessel occlusion (neointimal index, NI). Graft vessels showed endothelialitis in the RCMV-infected, but not in the uninfected, allogeneic recipients at 7 and 14 days post-op. TVS was detected at 21 days post-op with little difference between infected and uninfected allogeneic recipients (NI = 38 vs NI = 35; p = ns). However, at days 28, 35 and 45 post-op, RCMV infected recipient heart graft vessels showed a dramatic increase in the severity of TVS (NI = 49, 64 and 82) compared to uninfected allografts (NI = 31, 30 and 43; $p < 0.001$). These data suggest that the effect of RCMV on acceleration of TVS is manifested between 21 and 28 days post-op. Using quantitative PCR specific for the viral polymerase sequences, RCMV DNA was detected in both the graft and native hearts at all times tested, whereas, viral DNA was only found in the blood up until 14 days.

In order to investigate what effect RCMV infection has on chemokine expression kinetics, we determined chemokine expression in graft ± RCMV infection using quantitative RT-PCR (TaqMan) techniques. Chemokine profiles in graft hearts dramatically differ with or without RCMV infection. In the infected animals, allografts showed high expression of the CC-chemokines RANTES, MCP-1 and MIP-1α between 21 and 32 days post transplantation. Interestingly, the CXC chemokine IP-10 and the cytokine interferon gamma, which is the cytokine that drives IP-10 expression, were both increased about 20-fold at day 28 in the infected allografts compared to uninfected controls. Expression of two other chemokines Lymphotactin (C chemokine) and Fractalkine (CX3C chemokine) was also increased between days 21 and 32 corresponding to the timing of RCMV-accelerated TVS.

To determine whether the RCMV-induced chemokine expression causes a similar enhancement of immune cell infiltration into graft vessels, the subset and distribution of infiltrating leukocytes was analyzed by immunohistochemical staining of either frozen or paraffin sections of allograft hearts harvested at POD 24, 28, and 32. Serial sections were stained for ED I (macrophage/monocyte marker), CD4 (T helper cell marker), or CD8 (cytotoxic T cell marker). As expected, native hearts from either infected or uninfected recipients failed to stain for EDI, CD4 or CD8. Vessel staining for the macrophage marker EDI was minimal in sections from uninfected allografts until day 32 post-transplantation. However, grafts from RCMV-infected recipients showed faint staining for ED I as early as 24 days post-transplantation with moderate to intense staining for macrophages at days 28 and 32. Similarly, uninfected controls demonstrate little or no CD8 T cell staining at days 21 and 28 post-transplantation but at day 32 there is a slight increase in CD8 T cell staining, which was less intense compared to vessels from RCMV-infected allografts. The infected grafts demonstrate the presence of $CD8^+$ T cells at days 24, 28, and 32 (staining ranges from faint to moderate). Little or no vessel $CD4^+$ T cell staining was observed at all times post-transplantation in the uninfected allografts. However,

at day 32 the $CD4^+$ T cell staining of vessels from the infected allografts was moderate to intense. These findings demonstrate that RCMV-infected allograft vessels contain increased cellular infiltration compared to uninfected controls.

The RCMV within the allografts induces early events (endothelialitis), as well as upregulation of chemokine expression throughout the development of TVS leading to increased allograft recruitment of inflammatory cells. These events culminate in the RCMV-acceleration of this disease process. Allograft chemokine expression profiling demonstrates that RCMV infection dramatically enhances host chemokine expression with kinetics that match the timing of the virus-mediated acceleration of TVS observed in this model. In the third year, we will use both a panel of antibodies to different kinetic classes of RCMV-encoded genes and RCMV-genome arrays, to map the virus gene expression during the process of CMV-accelerated chronic rejection.



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Donor Peripheral Blood Mononuclear Cells Homing the Thymus of Recipients to Induce Graft Tolerance

The project was designed with the following specific aims:

1) To evaluate the effectiveness of infusion of donor dendritic cells (DCs) transduced with CD44 and/or CCR9 genes to induce kidney allograft tolerance in rats
2) To assess whether homing the thymus of rat recipients after infusion of donor DCs transduced with CD44 and/or CCR9 is necessary for graft tolerance induction
3) To explore possible mechanism(s) of long-term donor-specific allograft survival, if any, induced by infusion of donor CD44 and/or CCR9-transduced DCs
4) To investigate the specific contribution of intrathymic clonal deletion to the tolerogenic effect of donor CD44 and/or CCR9-transduced DCs in cardiac transplanted mice

Growth and characterization of mature and immature rat dendritic cells

DCs were obtained from rat (Brown Norway, BN) bone marrow (BM). To obtain mature DCs, BM cells were grown for 9 days in RPMI supplemented with rGM-CSF (100 U/ml), interleukin-4 (20 ng/ml) and FCS (10%). At day 9, dislodged cells were centrifuged and re-cultured for 36 hours with RPMI-FCS and growth factors, followed by 16 hours with FCS-RPMI. Cells were collected and either analysed by FACS or used for MLR. FACS analysis (Table 1) showed that most cells are mature DCs, $CD11c^+$, MHC II^+, $B7\text{-}2^+$, and express CD44 antigen. In 5-day one-way mixed lymphocyte cultures, irradiated mature DCs strongly stimulated the proliferation of allogeneic Lewis lymph node cells (Table 2).

	Mean fluorescence intensity	
	Mature DCs	Immature DCs
$OX6^+$ (MHCII)	79 335	72 193
$CD11c^+$	76 50	79 77
$B7\text{-}2^+$	77 903	72 539
$ED3^+$ (macrophages)	13 16	7 18
$CD44^+$	87 112	75 156

Table 1. *FACS analysis of rat bone-marrow-derived DCs*

	[^{3}H] Thymidine uptake (cpm)	
DC:LN	Mature DCs	Immature DCs
1:30	72 360	1222
1:100	51 190	9 853
1:1000	155 060	135 770
1:10 000	28 380	49 150

Table 2. *MLR allostimulatory activity of rat bone-marrow-derived DCs. The mean [^{3}H]thymidine uptake of syngenic BN lymph nodes (LN) cultured with BN DCs was about 1400 cpm.*

To obtain immature DCs, BN BM cells were grown as above except that rat serum was used instead of FCS to avoid stimulation by xenogenic proteins. The cells obtained at the end of the 11-day culture expressed lower levels of MHC II and B7-2 than mature DCs grown in the presence of FCS. In contrast, CD11c and CD44 expression levels were comparable in mature and immature DCs (Table 1). In 5-day one-way mixed lymphocyte cultures, irradiated immature DCs stimulated the proliferation of allogeneic Lewis lymph node cells less efficiently than mature DCs (Table 2).

Effect of pre-transplant infusion of donor dendritic cells on kidney graft survival

To evaluate the effect of pre-transplant infusion of donor BM-derived DCs on the survival of a subsequent kidney allograft from the same donor strain, two Lewis rats were infused with either mature (rat 1) or immature (rat 2) BN-BM-derived DCs (6×10^6 cells each). The animals were given a cyclosporine A (CsA) for 10 days (10 mg/kg), then CsA was stopped and the animals received a BN kidney transplant. Grafts survival was as follows (rat 1, 9 days; rat 2, 16 days), indicating that donor immature but not mature DCs are capable of slightly prolonging allograft survival (mean graft survival in control transplanted rats pre-treated with a 10-day course of CsA = 8 days).

Since FACS analysis has shown that the rat BM-derived DCs express high levels of CD44, we concentrated our efforts on cloning the rat CCR9 and obtaining a corresponding adenoviral vector with the goal to transfect the CCR9 cDNA into BM-derived DCs. This should enhance their migration into the thymus, and possibly increase their tolerogenic properties.

Cloning of rat CCR-9

Since the rat CCR9 gene had not yet been described, we began to obtain the cDNA sequence. RNA from rat thymocytes was extracted with TRIzol reagent. 5 µg of RNA were primed with oligo(dT) and reverse transcribed to cDNA. Primers (forward: 5'-GGATGTTCCAGACCTTCATG-3'; reverse: 5'-ATGAAACCCACTGGGCCT-3') were designed in a conserved region of human and mouse CCR9 (human: XM003251.1; mouse: AJ132336). The obtained PCR product (as expected 702 bp in length) was sequenced and showed an open reading frame with a translated sequence that was 97% and 86% identical to those of the corresponding mouse and human CCR9, respectively.

Specific primers were designed for rapid amplification of cDNA ends to obtain the 5' and 3' ends of the transcript. The RACE-PCR products were ligated into a pGEM T-Easy vector and cloned in *E. coli*. Independent clones were sequenced to obtain a consensus sequence. Using overlapping 5'- and 3'-RACE sequences, the entire rat CCR9 gene sequence (2568 bp), including the coding region and flanking untranslated regions, was deduced. Alignment of the rat, mouse and human CCR9 amino acid sequences revealed high homology among the species (97% amino acid identity rat/mouse; 86% amino acid identity rat/human). The 78 C-terminal residues are the same in all species. In contrast, in the untranslated regions, cDNA sequence homology among rat, human and mouse CCR9 is low, particularly at the 3' end of

the transcript. The rat transcript is about 250 bp shorter than the corresponding mouse sequence: however, since we did not identify a polyadenylation signal, we cannot exclude that longer transcript exists.

At the 5' end, the rat transcript is smilar to human CCR9A. A second transcript, CCR9B, 49 bp shorter than CCR9A due to an alternative splicing, has also been reported in humans (Yu et al. *J Immunol* 2000; 164:1293-1305) but not in mice. To evaluate whether the B form of CCR9 RNA is expressed in rat, a short PCR fragment, overlapping the alternative splicing was amplified. However, we only obtained the fragment corresponding to the longer CCR9A transcript.

The rat CCR9 sequence we obtained was submitted to GenBank under the accession number AF458780: at present it is still held confidential.

Analysis of CCR9 expression

CCR9 expression in several rat cell types was analysed by real-time quantitative PCR. Total RNA was extracted from total thymocytes, PBMC, splenic APCs (a mixture of macrophages and DCs) and BM-derived DCs (either immature or mature as defined above). RNA, previously treated with DNase, was primed with random examers and reverse transcribed to cDNA.

A fluorescent-labelled probe (5'-TTCACAAGCCTTATTCCTGGCATGTTTGA-3') and sense (5'-GCGTCTGACCCACCATGAC-3') and antisense (5'-GGAAGCAGTGGAATCATAGCTGTA-3') primers were designed based on the rat CCR9 sequence. Since the probe spanned an exon junction, genomic DNA was not amplified, as documented by negative reverse transcription controls that showed no signal. To assess the overall cDNA content, GAPDH served as the housekeeping gene. Quantitative PCR was performed in triplicate. Amplification efficiencies for target and housekeping genes were demonstrated to be similar by analysing serial cDNA dilutions. Thus, we could use the technique to compare the cDNA content of each sample with another sample, taking the cDNA expression in total thymocytes as a reference.

Cell type	Ct CCR9	Ct GAPDH	ΔCt	ΔΔ Ct	2^ -ΔΔCt
Total thymocytes	25.27667	22.81333333	2.463333	0	1
PBMC	30.11333	20.88	9.233333	6.77	0.009162773
Splenic APCs	27.71333	20.29	7.423333	4.96	0.032128557
Mature DCs	29.63333	18.56666667	11.06667	8.603333	0.002571216
Immature DCs	28.78667	17.56	11.22667	8.763333	0.002301303

Table 3. *CCR9 expression in rat cell types analysed by real-time PCR.*

As expected, the expression of CCR9 in total thymocytes is very high with respect to all the other cell types examined (Table 3). In particular, the level of CCR9 expression in BM-derived DCs was about 500 fold lower than in total thymocytes. No difference in CCR9 expression was found between immature and mature BM-derived DCs.

Generation of E1/E2a/E3-deficient adenoviral vector encoding the rat CCR9 gene

The entire CCR9 rat coding sequence (1166 bp) was amplified with the following primers: forward 5'-CCTGAAGCTGACTGGCGTC-3'; reverse 5'-AGGACCATATGCCCAGTGAGA-3'. PCR

was performed on rat thymus cDNA. The PCR product was ligated into a pCR2.1 vector and cloned in JM109 bacterial cells. Individual clones were screened by sequencing to find a plasmid with no errors introduced by PCR. A correct clone was selected and subsequently subcloned into a pBSitrLxMCS vector.

To generate the Ad3-CCR9 we used a method involving recombinase-mediated recombination of two plasmids: pSQ3, containing the right hand portion of the adenovirus serotype 5 genome; and the shuttle plasmid, pBSitrLxMCS, containing the left end of the viral genome and the CCR9 expression cassette. Both plasmids contain one loxP site. pSQ3 and the shuttle plasmid were cotransfected into S8 cells (A549 cells stably transfected with the adenovirus E1 and E2a regions under the control of dexamethasone-inducible promoters) together with a plasmid encoding for the Cre recombinase (pCre). Production of Cre recombinase mediated the recombination between the loxP sites on the other two plasmids.

One day before homologous recombination, S8 cells were seeded on a 6-well plate and expression of E1 and E2a was induced by adding dexamthasone (0.5 mM) to the media (MEM/10% FBS). The linearised shuttle plasmid containing the CCR9 cDNA was cotransfected with the linearised pSQ3 and the pCre plasmids. The next day, cells were given fresh medium containing dexamethasone. Cells were harvested 7 days post-infection, spun down, and the pellet suspended with the collected medium, thus obtaining the crude viral lisate (CVL). The day before cell harvesting, new cells were plated on a 6-well plate and induced by dexamethasone. The pellet from the harvested cells was frozen and thawed three times, centrifuged to remove cell debris, and the supernatant was used to infect a new well of dexamethasone-induced cells. Cells were monitored for cytopathic effect (CPE). When full CPE was achieved, cells were harvested, and a new CVL obtained. After four cycles of freezing/thawing, serial dilutions of supernatant were used to infect new dexamethasone-induced cells, seeded the day before on a 6-well plate. The medium containing the serial dilution of the virus was left overnight and on the next day cells were overlayed with 3 ml agarose (2%)/EMEM (2x) to obtain single plaques. When evident, single plaques were picked up in 400 ml complete medium, frozen and thawed four times, and the virus obtained from each plaque amplified on a 35-mm well. When full CPE was achieved, cells were harvested, spun down and the pellet suspended in 400 ml PBS. 200 ml were used to isolate genomic DNA to perform PCR analysis, to check the presence of the insert, and for sequencing and restriction digests; the other 200 ml were used to infect a 150-mm plate. When complete CPE was obtained, CVL were used to infect 10 150-mm plates and the CVL obtained from this amplification used to infect 80 150-mm plates. Finally, the CVL was CsCl purified, dialyzed, titrated and stored at -80°C.

Figure 1 shows PCR analysis from genomic DNA obtained from plaque 1, which used to amplified the virus, using primers specific for the vector sequence flanking the CCR9 cDNA. The CCR9-specific amplified band is 1378 bp long.

AGAACCCACAGAAGCATACTCATTCCAGCAGCTGCAGTGGTCTTTTCCCCACAGACCCAGGCTCTGCACT
TCCCCTCCTGAAGCTGACTGGCGTCTGACCCACC**ATG**ACACCCACAGAATTCACAAGCCTTATTCCTGGC
ATGTTTGATGACTACAGCTATGATTCCACTGCTTCCACAGACGACTACATGAATTTGAACTTCAGTAGCTTCT
TCTGTAAGAAAAACAATGTAAGGCAGTTTGCGAGTCATTTCCTTCCGCCTCTGTACTGGCTTGTGTTCGTTGT
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ATGTTCCTTTTGAATTTGGCCATTGCTGATCTGCTCTTTCTTGCCACTCTTCCCTTCTGGGCCATTGCTGCTGCT
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GTGCTACTCATCATGTGCATCAGTGTGGACAGATACATTGCCATTGTACAGGCCATGAAGGCTCAGGTCTGG
AGGCAGAAAAGGCTGCTGTACAGCAAGATGGTCTGCATTACCATCTGGGTGATGGCAGCTGTGCTCTGCAC
CCCAGAAATCCTGTACAGTCAAATCAGTGGGGAATCTGGCATTGCCATATGTACCATGGTCTACCCTAAGGA
TAAGAATGCCAAGCTAAAGTCTGCTGTTTTGATCCTGAAGGTCACCTTGGGGTTTTTCCTCCCCTTCATGGTC
ATGGCTTTCTGTTACACCATCATCATTCATACTTTGGTACAGGCCAAGAAGTCATCCAAGCACAAGGCCCTC
AAGGTGACCATCACTGTTCTCACTGTCTTCATTATGTCTCAGTTCCCCTACAATTGCATTCTGGTAGTGCAGGC
TGTTGATGCCTACACCATGTTCATCTCCAACTGCACCATTTCCACCAACATTGACATCTGCTTCCAGGTTACT
CAGACTATTGCATTCTTCCACAGTTGCCTGAACCCAGTTCTCTATGTTTTCGTGGGTGAGAGATTCCGCAGG
GATCTGGTAAAGACCCTGAAGAACCTGGGATGCATTAGCCAGGCCCAGTGGGTTTCATTCACAAGGAGAG
AGGGTAGCTTGAAGCTTTCTTCTATGCTACTGGAGACAACTTCTGGAGCTCTCTCCCTG**TGA**GAGAACTTCT
CACTGGGCATATGGTCCTTTTTGGAAATAATGAGATATATAGACACAGCATAATCTAAAGAGACAGCAAACA
GAAAGAAAACGGGGGGAGGCGGCTGGGGGAGGCAGGAAAAAACAAAACAAACAACTGGAATGAACC
TAAACAACTTTATCAAGTTCAGTTAGAATCTACCAAAAGTTTCCAAAACTGACCACCCCAGAACGGCACC
AGCTGGTTTTTGGTCACAGTAACTGTAGTTCTGAGGAGGATACTTGAGCAGCAGTGAGGACACTCCAGCTT
TGCACAGGCTGATCATCAGTGCAGCTAGGTTTTGGAGGCATTGAGTTTCTTCAGTGCTCCAAGTTTCTGTTAC
TTCAGATCTGGTGCTACTCCTCTCCAGAGGTGACAGAGTACTGGCTGCCACCATACCCCCTTTCCCCCCAC
CAAAAGCTTAGTGACATCCACCTCAGGATCTTTTCTTTATACTCCTACTGGTGGAAAGACTGTGTTTGTATGTC
TCATTACCTATAGTTTTGTTTTTGTTTTTGTTTTTGTTTTTAAATCTAGAACACACTGAAACATTCTGAGGCTGA
ATGCCATTCTGATCTCCTTGGTCTCTTCTGAGCCAGGGAGAATCACTGTTCCTCGTTTGAACCATCTGTAAGG
TTGGTCAGTCCATGTATAGCTGACCACATCACATTACCTGGATTCTTGCTCCCAGTCCACTTCTGTGTCAGGC
AGAAGATGCCATGTCCAAACCTCACTGCAAGCACAAACTAGGAAAGGATCTAGCCTGTTCCTGGGTGTGG
TGAAAGTGGCCCTCACAGAATTAAGCCGGAGAATATGTTGGGATGGTGGGGACCTAGCCTTTGGGACTTC
CTTAGGGAAGAGGCACTTGCTGCCGGTTTTCCCATCTTCTTCTTACCAGTGAACTTCTGAGGCTCCCGGAA
GGGTGTCTGCCTCCCTGCTGCCTCCCCTTTCCCTACTTTGTAGTCTTGACAGTTTACAACCTTTTAATGTTAGA
TTAAGAAGAATGGGTGGGTTGGGCATGATAGTGTACACCTTTTATCCCAGCAGAGGCAGGGTGATCTCTGT
GAGTTGGAGGCCAGCCTGGTCTACAGATGGAGTCCAGGAGATTCGGGGCTGCTTATGCAGAGAAATCTCA
TCATGAAAAACAAACAAACAAAAAGTAAAAAAAGGGTTGAAAAGATGGCTCAATGGTTAAGAGCACTGA
CTGCTCTTCCCGAGGTCCAGGGTTCAATTCCCAACACCTACATGGCAGCTCACAACTGTCTGTAACTTCAGT
TGCAGAGGATCTGATACTCTCACACAGACACACATGCAGGCAAAGCACCAATGCACATG

Figure 1. *Rat CCR9 gene sequence. Start and stop codons are shown in bold.*

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Cytokine-Activated Signaling in T Cells is Required for Tolerance Induction by Allochimeric Protein

The goal of our study is to understand the mechanism of transplantation tolerance induced by designed donor/recipient class I major histocompatibility complex (MHC) antigens ($\alpha_{1h}{}^{u}$70-77-RT1.A^{a} protein). Our previous results showed that $\alpha_{1h}{}^{u}$70-77-RT1.A^{a} protein alone (bearing four donor polymorphic amino acids; a.a. His^{70}, Val$_{73}$, Asn$_{74}$, and Asn$_{77}$) induced tolerance to Wistar Furth (WF; RT1^{u}) heart allografts in ACI (RT1^{a}) recipients. In addition, oral delivery of $\alpha_{1h}{}^{u}$70-77-RT1.A^{a} protein alone also induced tolerance and prevented chronic rejection. Adoptive transfer experiments confirmed that tolerance is maintained by potent regulatory T cells. Finally, tolerance coincided with reduced numbers of interleukin (IL)-2/interferon (IFN)-γ-producing T helper (Th1) cells and elevated numbers of IL-4/IL-10-producing Th2 cells. The same tolerant T cells displayed diminished TCR-driven signaling via extracellular regulated kinase (Erk), AP-1, and NF-κB, as well as the common γ-chain (γ_c) cytokine-receptor-induced signaling by Janus kinase 3 (Jak3)/stimulators and activators of transcription (Stat)5 pathways.

HYPOTHESIS 1: *Effective IL-4/Stat6-driven upregulation of Th2 is required for induction of transplantation tolerance by allochimeric protein.* During the second year of funding we have confirmed that induction of transplantation tolerance by allochimeric protein depends on IL-4-driven T helper 2-type regulatory cells. Ten highly polymorphic amino acids (a.a.) were found in α1 (α_{1h}), but not in α2 (α_{2h}), helical regions of the RT1.A^{n} rat class I MHC alloantigen. The allochimeric proteins, with ten donor-type polymorphic a.a. in α1 helical region ($_{1h}\alpha_{1h}$-RT1.A^{n}) or α2 helical region (α_{2h}-RT1.A^{n}) produced in an *Escherichia coli* expression system, were tested for their potential to induce accelerated rejection or tolerance. The *in vivo* results documented that a single portal vein administration of α_{1h}52-90^{n}-RT1.A^{c} protein, induced tolerance to BN (RT1^{n}) heart allografts in PVG (RT1^{c}) recipients more effectively than wild-type (WT)-RT1.A^{n} protein; α_{2h}148-179^{n}-RT1.A^{c} protein was ineffective. In contrast, subcutaneous injection of WT-RT1.A^{n} (but neither α_{1h}52-90^{n}-RT1.A^{c} nor α_{2h}148-179^{n}-RT1.A^{c}) protein induced accelerated rejection of BN heart allografts. Thus, allochimeric proteins are highly tolerogenic and only slightly immunogenic.

We evaluated the spectrum of cytokines using an ELISA spot assay, namely, the number of IL-2- and IFN-γ-producing Th1 cells and IL-4- and IL-10-producing Th2 cells. Untreated recipients showed a marked increase in the numbers of both Th1 and Th2 on days 5 and 7

post-grafting; a pattern that correlated with allograft rejection. In contrast, a single injection of $\alpha_{1h}52\text{-}90^{n}$-RT1.A^{c} protein induced an early (days 5 and 7 postgrafting) increase in the number of Th2 cells, and a reduction in the number of Th1 cells. However, tolerant recipients treated with the $\alpha_{1h}{}^{n}$-RT1.A^{c}/CsA protocol displayed a long-term reduction of Th1 cells and a persistent elevation of Th2 cells (days 5, 7, 14 and 200 post-grafting). Thus, down-regulation of Th1 cells and selective activation of Th2 cells correlates with induction and maintenance of tolerance by allochimeric proteins. The poorly immunogenic $\alpha_{1h}52\text{-}90^{n}$-RT1.A^{c} allochimeric protein induces tolerance by selective activation of regulatory Th2 cells.

Using these two *in vivo* models (WF to ACI and BN to PVG) we plan (during the third year) to show that tolerant recipients have predominantly IL-4-driven Stat5/ Stat6-dependent Th2 cells. These experiments will be performed by electroporetic mobility shift and supershift assay (EMSA). Furthermore, a panel of T cell clones generated in the first year of this project will be used in the third year to examine the pattern of activation of Stat5 and Stat6 transcription factors (EMSA and Western blot).

HYPOTHESIS 2: Delivery of a full, second B7/CD28 signal, is needed to induce transplantation tolerance by allochimeric protein. Our previous results documented that a second signal (B7/CD28) is needed to induce regulatory T cells that maintain transplantation tolerance. In particular, induction of tolerance by administration of $\alpha_{1h}{}^{u}$70-77-RT1.A^{a} protein alone was blocked by treatment with CTLA4-Ig (blocking signal 2). Additional *in vitro* experiments revealed that blockade of signal 2 prevented generations of IL-4-driven Th2 cells. In similar fashion, induction of tolerance by administration of $\alpha_{1h}{}^{u}$70-77-RT1.A^{a} protein alone was blocked by rapamycin (blocking the mammalian target of rapamycin, mTOR) or by AG490 (targeting Jak3) as well as preventing generation of Th2 regulatory cells. Thus, generation of regulatory IL-4-driven regulatory Th2 cells requires both signals 2 and 3.
In the third year we plan to examine the role of signal 2 in signaling through Jak3/ Stat5/Stat6 pathway.

HYPOTHESIS 3: Apoptosis of alloantigen-specific T cells (AICD) is not necessary for induction of transplantation tolerance by allochimeric protein. Our preliminary experiments documented that 6-carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled T cells may be used for *in vivo* experiments. During the third year we plan to prove that AICD is not needed for induction of tolerance by allochimeric proteins. To perform this experiment, the ACI T cells labeled with CFSE will be injected into irradiated ACI recipients of irradiated WF spleen allografts. On days 2 or 5 after injection, spleen cells will be examined for the number of divisions (steptoavidin-Cy-Chrome CD4 or CD8 Ab) and for AICD (PE-conjugated annexin V-specific antibody). Using this system AICD will be also evaluated in animals that are treated with tolerogenic protein alone or in combination with CsA, rapamycin, or AG490. These experiments will help to establish whether AICD is necessary in our model. We postulate that AICD is not necessary for induction of tolerance by allochimeric protein.

Publications

1. Stepkowski SM, Kirken RA, Trawick B, Wang M, Tejpal N, Wang ME, Tian L, Clark J, Kahan BD. Allochimeric class I MHC protein-induced tolerance by partial TCR engagement requires activation of both CTL4- and common γ-chain-dependent cytokine signals. *Transplantation* 2002, in press.
2. Perez J, Stepkowski SM, Trawick B, Wang M-E, Kahan BD. Selection of low immunogenic and highly tolerogenic allochimeric class I MHC proteins. Manuscript in press.
3. Stepkowski SM, Kirken RA, Nagy ZS, Trawick BW, Wang M, Tejpal Nm Wang ME, Tian L, Clark J, Kahan BD. The role of Stat5 in the induction of regulatory T cell in transplantation tolerance. *Transplant Proc* 2001; 33:3835.
4. Stepkowski SM, Nagy ZS, Kahan BD, Kirken RA. The role of signals 1, 2 and 3 in induction of transplantation tolerance. *Transplant Proc* 2001; 33:3831.
5. Wood KJ, Stepkowski SM. Transplantation tolerance—discussion. *Tranplant Proc* 2001; 33:3819.
6. Stepkowski SM, Kirken RA, Wang M, Akioka K, Kahan BD. Tolerance induction by alteration of T cell receptor (TCR) signaling using an allochimeric donor/recipient class I MHC protein. *Transplant Proc* 2002; 33:131.
7. Kirken RA, Stepkowski SM. New directions in T cell signal transduction and transplantation tolerance. *Curr Opin Organ Transplant* 2002; 7:18-25.

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Mechanisms Involved in Prevention of Donor-Specific Cardiac Allograft Rejection by Regulatory T Cells

Transplantation is the current therapeutic modality for patients with end-stage organ failure or haematopoietic malignancies. Joint efforts of clinicians and immunologists have now made it possible for 80-95% of patients to live with a functional allograft for one year post-transplantation. Nonetheless, despite vast improvements in 1-year graft survival rates, long-term graft survival has remained relatively unchanged over the past 30 years.

Long-term immunosuppression with drugs, such as cyclosporine, is the most commonly used approach for enhancing allograft survival. However, many immunosuppressive drugs currently in use are much less robust in preventing long-term graft rejection than short-term graft rejection. The advantage of cyclosporine treatment diminishes beyond one year, as evidenced by only a small increase (one year) in half-lives of graft survival. Even with continuous immunosuppressive treatment, half of the patients receiving transplants lose their grafts in five years. In addition, as immunosuppressive drugs inhibit immune responses non-specifically, their use results in a high incidence of infectious and malignant complications in these recipients. Hence, the induction of donor-specific tolerance in the absence of expensive, toxic and non-specific immunosuppressive therapy has been a goal of transplantation for many years.

Numerous studies indicate that under appropriate conditions, pre-exposure of an individual to a specific antigen may lead to tolerance to that antigen rather than immunization. Intravenous injection of lymphoid cells carrying specific transplantation antigens identical to the graft donor, including donor lymphocyte infusion (DLI), is one such approach used to induce tolerance to alloantigens both in man and rodents. Unlike immunosuppressive drugs that non-specifically inhibit immune responses, DLI reduces the specific immune response to the donor antigen without impairing immune responses to other antigens, thereby avoiding the complications of infection and malignancy generated by using immunosuppressive drugs. Despite the fact that the beneficial effect of DLI on long-term allograft survival is a phenomenon has been observed for nearly 30 years, the mechanisms whereby the introduction of donor cells lead to tolerance are still not fully understood, which limits the application of DLI in transplantation.

My laboratory has developed a protocol by which long-term donor-specific skin allografts can be achieved by pre-transplant DLI without the need for any non-specific toxic immunosuppressive drugs. We have demonstrated that there is a significant increase in the number of mature

$\alpha\beta^{-}TCR^{+}CD3^{+}CD4^{-}CD8^{-}$ double negative (DN) T cells in the accepted grafts and tolerant recipients. DN T cells comprise 1-5% of peripheral lymphocytes in rodents and 1-2% of human peripheral blood lymphocytes. We have demonstrated that DN T cells in DLI-treated mice have a potent immune regulatory function, and can specifically down-modulate allogeneic immune responses *in vitro* and *in vivo*. We hypothesized that DLI given in certain ways can promote tolerance induction in both allogenic and xenogenic heart transplantation models. Recipient-derived DN regulatory T cells play an important role in induction of donor-specific transplantation tolerance.

The goal of this project sponsored by ROTRF was to determine the role and mechanisms of DN regulatory T cells in the induction and maintenance of donor-specific cardiac transplant tolerance. The specific objectives were:

1) To develop a model in which long-term survival of cardiac grafts can be achieved by DLI

2) To determine the role and mechanism of DN regulatory T cells in prevention of donor-specific cardiac allo- and xenograft rejection

3) To determine the role of adhesion molecules in the recruitment (emigration) of DN regulatory T cells to cardiac grafts and lymphoid organs

4) To identify specific and selective cell surface markers for DN regulatory T cells

During the initial funding period (10 months), we have made the following progress:

1) We have developed a model in which long-term (>200 days) cardiac xeno-graft survival can be achieved by combination treatment with pre-transplant DLI and a short course of depleting anti-CD4 monoclonal antibody

2) We have demonstrated that the number of DN T cells increases in recipients that have accepted cardiac xenografts

3) We demonstrated that the recipient-derived DN T cells suppress the proliferation of anti-donor responses mediated by naive $CD4^{+}$ and $CD8^{+}$ T cells *in vitro* in a dose-dependant manner

Our preliminary results suggest that DLI alone can induce long-term survival of a heart allograft with one MHC class I mismatch. Pre-transplant DLI together with a short course of depleting anti-CD4 monoclonal antibody can significantly prolong fully MHC-mismatched cardiac graft survival.

Our preliminary studies in humans suggest that DN regulatory T cells exist in humans, which can downregulate immune responses *in vitro*. The results obtained from these studies allow us to further investigate the mechanisms involved in the induction and maintenance of cardiac allo- and xenograft survival, and will be published in the near future.

5. Final Reports of ROTRF Grantees

Dr. Michael Autieri, Principal Investigator
Christopher Carbone, Research Associate

Temple University, Philadelphia, USA

Characterization of Expression and Growth-Enhancing Properties of AIF-1 in VSMC of Injured Arteries

The specific aims of this proposal were to

1) define the mechanism of AIF-1 growth-enhancing activity in human vascular smooth muscle cells (VSMC),
2) characterize the expression pattern of AIF-1 and its sensitivity to various therapeutic compounds, and
3) explore its use as a surrogate marker of VSMC pathophysiology.

Only the first aim of the grant was funded. Thus, we have focused our efforts on determining the growth-enhancing effects of AIF-1 on human VSMC by several approaches.

Retroviral transduction of human VSMC with AIF-1

We generated retrovirus (RV) containing the AIF-1 protein-coding region cDNA and empty vector RV as controls. Stable transfectants were isolated by antibiotic selection. Over-expression of AIF-1 was verified by Western blotting (Figure 1).

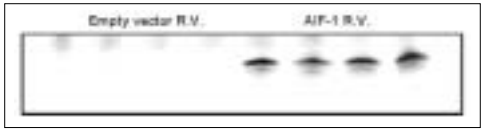

Figure 1. *Over-expression of AIF-1 protein in stably transfected primary human VSMC. Extracts from four independent transductions were separated by SDS-PAGE, and AIF-1 content determined by Western analysis with AIF-1 antibody.*

We found that human VSMC that stably over-express AIF-1 grew more rapidly than control cells (Figure 2). This held true both in the presence and absence of serum growth factors. This suggests that AIF-1 overexpression allows VSMC to grow in the presence of reduced growth factors, and allows VSMC to respond to growth factors in an enhanced fashion.

We attempted to ascertain a mechanism for these growth effects. First, we isolated mRNA from stably transduced VSMC, and performed cDNA microarray analysis to determine whether AIF-1 overexpression influenced gene expression. The results shown in Figure 3 indicate that the mRNA for two cell cycle genes, cyclin-dependent kinase 2 (Cdk2) and cyclin-dependent kinase 6 (Cdk6), are increased 11-fold and 8.5-fold, respectively, in serum-starved AIF-1-containing cells. This result still requires verification by Western blotting, but nonetheless suggests a potential mechanism for AIF-1 growth effects in serum-reduced medium.

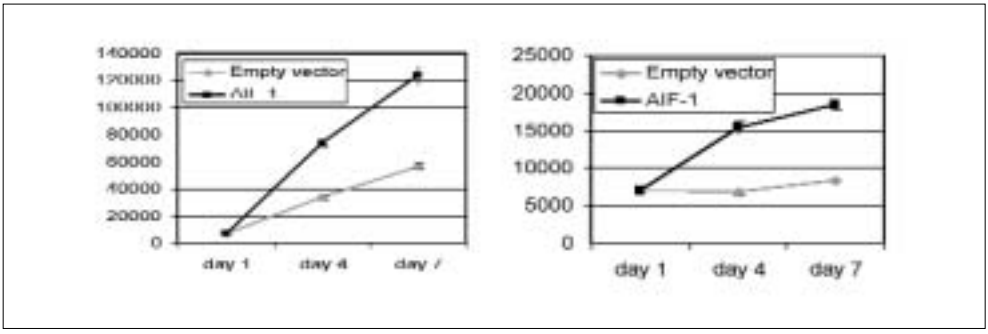

Figure 2. *Primary human VSMC that stably over-express AIF-1 grow more rapidly than those that do not. Equal numbers of pooled human VSMC stably transduced with AIF-1 retrovirus or empty vector were seeded into 12-well plates and grown in growth medium (A), or 0.3% fetal calf serum (B). After 1, 4 and 7 days, cells were trypsinized and counted in triplicate. Numbers on the y-axis indicate cells per well. A representative experiment is shown from three independent transfections with similar results ($p<0.01$).*

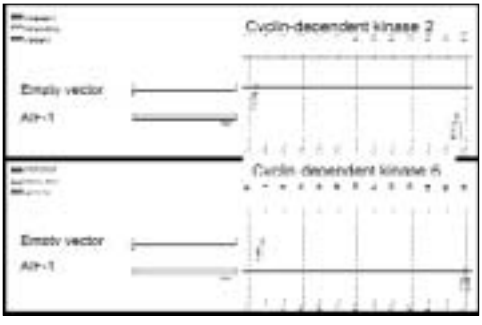

Figure 3. *Expression of cell cycle genes in stably transduced human VSMC. Cells were transduced with either AIF-1 or empty vector RV, and stable transductants isolated by antibiotic selection. VSMC were serum starved for 48 hours in 0.3% fetal calf serum, and total RNA isolated. Gene expression was determined by cDNA microarray analysis. Quantitation and fold increase were determined by the GeneExpress program.*

Results from cDNA microarray analysis also suggested that the mRNA for the transcription factor early growth-responsive gene-1 (Egr-1) was also upregulated in AIF-1-containing cells. Egr-1 mRNA and protein is normally expressed one hour after cytokine stimulation, and then declines within four hours. Our experiment demonstrated that Egr-1 protein was present as late as 24 hours post-stimulation (Figure 4), pointing to either a stabilization of growth-factor-induced

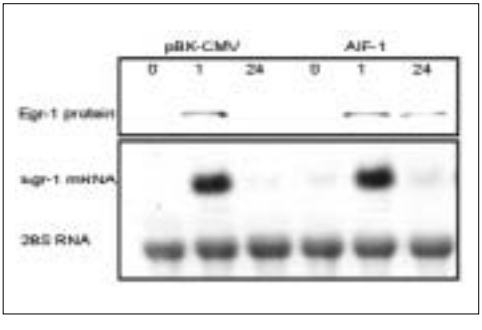

Figure 4. *Effect of constitutive AIF-1 over-expression on Egr-1 mRNA and protein expression. Human VSMC stably transduced with empty vector or AIF-1 retrovirus were serum-starved for 48 hours, then stimulated for 1 hour and 24 hours with 10% FCS. A. Protein extracts were separated by SDS-PAGE, transferred to nitrocellulose, Western blotted with Egr-1 antibody, and detected by the chemiluminescence method. B. Total RNA was extracted from the samples described above, probed with an Egr-1 cDNA probe by Northern analysis, and detected by autoradiography.*

early activation proteins, or a sustained expression or stabilization of early activation mRNA. Figure 4 shows a typical Egr-1 mRNA expression pattern in control and AIF-1-expressing cells, where Egr-1 mRNA is only expressed in cells stimulated with serum for one hour, but not in unstimulated or 24-hour-stimulated populations. This mRNA expression pattern, in conjunction with the Egr-1 protein expression pattern, suggests that AIF-1 over-expression leads to stabilization of Egr-1 protein, or protects it from degradation.

This is potentially a very exciting finding, as Egr-1 is an early growth factor and stress-responsive transcription factor induced in vascular cells in response to arterial injury[1,2]. The promoter regions of many genes that encode mitogenic and survival factors expressed by activated VSMC contain Egr-1 regulatory sites, including those for PDGF, TGF-β, FGF-2, TNF-α and p53. Further, inhibition of Egr-1 mRNA inhibits VSMC proliferation and balloon anigoplasty-induced restenosis[3]. These data may provide the molecular link that explains the mechanism of AIF-1 growth enhancement of VSMC.

References

1. Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature* 1993; 362:801-809.
2. Khachigan LM et al. Egr-1-induced endothelial gene expression: A common theme in vascular injury. *Science* 1996; 271:1427-1431.
3. Santiago FS et al. New DNA enzyme targeting Egr-1 mRNA inhibits vascular smooth muscle proliferation and re-growth after injury. *Nat Med* 1999; 5:1264-1269.

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Prolonged Preservation of Donor Livers Using Normorthermic Perfusion

We have developed a means of preserving donor livers whereby the organ is perfused with blood and maintained at normal body temperature. This enables the liver to continue to function during preservation.

Initially we compared the function of porcine livers perfused with blood at body temperature for 24 hours with those stored for the same period with conventional preservation solution at ice temperature and subsequently reperfused at body temperature. In order to quantify the effects of preservation and reperfusion injury, metabolic, synthetic and histopathological parameters were carefully evaluated. All showed a dramatic advantage of warm blood preservation over the traditional cold flush technique.

In order to simulate the clinical situation of a non-heart-beating donor, we extended the study to incorporate livers that had sustained an ischaemic injury prior to preservation. The warm preservation technique was able to recover these livers, resulting in a functioning organ following 24 hours of preservation. In contrast, cold-stored organs, having sustained a period of warm ischaemia, were rendered non-viable.

We have also been working on developing a model of fatty change in the porcine liver, to provide a model of a pathology that is found in a large proportion of cadaveric donor livers. Although not yet reliably established, this should provide an important tool for further research in this area.

These results are extremely encouraging and demonstrate substantial advantages of this new technique when compared with the current practice of cold preservation. We propose that this warm preservation technique minimises both the preservation and the ischaemia-reperfusion injuries associated with cold preservation. This opens up the possibility of expanding the donor pool by enabling ischaemically damaged livers to be resuscitated during the preservation process. Before considering clinical trials, we plan to validate the results in a pig liver transplant model.

Publications arising from this work

Friend PJ, Imber C, St Peter S, Lopez I, Butler AJ, Rees MA. Normothermic perfusion of the isolated liver. *Transplant Proc* 2001; 33:3436-3438.

Imber CJ, St. Peter SD, Lopez de Cenarruzabeitia I, Piggott D, James T, Mcguire J, Saville S, Hughes D, Butler A, Rees M, Taylor R, Friend PJ. Advantages of normothermic perfusion over cold storage in liver preservation. *Transplantation* 2002; 73:701-709.

Imber CJ, St. Peter SD, Lopez de Cenarruzabeitia I, Friend PJ. The use of laser doppler as a non-invasive measure of ischaemia/reperfusion injury in the pig liver. *Transplant Proc* 2000; 33:3718-3719.

Imber CJ, St. Peter SD, Handa A, Friend PJ. Hepatic steatosis and its relationship to transplantation. *Liver Transplant* 2002; 8:415-423.

Imber CJ, St. Peter SD, Lopez de Cenarruzabeitia I, Lemonde H, Rees M, Butler A, Clayton PT, Friend PJ. Optimisation of bile production during normothermic preservation of porcine livers. *Am J Transplant.* In press.

St. Peter SD, Imber CJ, Lopez de Cenarruzabeitia I, Saville S, Hughes D, Friend PJ. Extended preservation of non-heart beating donor livers with normothermic machine perfusion. *Br J Surgy* 2002; 89:609-618.

St. Peter SD, Imber CJ, Lopez de Cenarruzabeitia I, Mcguire J, James T, Taylor R, Piggott D, Friend PJ. ß-galactosidase as a novel marker of ischaemia-reperfusion injury and a mechanism for viability assessment in liver transplantation. *Transplant Proc* 2001; 33:3753-3755.

St. Peter SD, Imber CJ, Friend PJ. Liver and kidney preservation by perfusion. *Lancet* 2002; 359:604-613.

St. Peter SD, Imber CJ, Lopez de Cenarruzabeitia I, Mcguire J, James T, Taylor R, Friend PJ. ß-galactosidase as a novel marker of ischaemia-reperfusion injury and a mechanism for viability assessment in porcine liver transplantation. *Liver Transplant* 2002; 8:21-26.

St. Peter SD, Imber, CJ, Lopez de Cenarruzabeitia I, Friend PJ. The non-heart beating donor: bridging the gap to the future. *Transplant Rev* 2002; 16:51-61.

St. Peter SD, Imber CJ, Kay J, Lopez de Cenarruzabeitia I, James T, Friend PJ. Hepatic control of acid-base homeostasis on an extracorporeal circuit: an indicator of ischaemic injury and organ quality in the porcine model. Submitted for publication.

Prof. Philip F. Halloran, Principal Investigator
Dr. Calvin Harley, Dr. Walter Funk, Dr. Nam Kim,
Dr. Kim Solez, Dr. Ron Moore, Dr. Gerald Todd

University of Alberta, Edmonton, Canada

The Role of Senescence and Telomere Shortening in Chronic Rejection

OBJECTIVE: Donor age is now as important as histoincompatibility in determining graft outcomes but the molecular basis of aging is unknown. Kidneys from older donors must be used because of the organ shortage. The present project looked at aging mechanisms to begin to understand donor age. In particular we asked whether cell senescence, which occurs in all somatic cells in culture, occurs in human kidneys and contributes to the aging phenotype. We selected several indicators of cell senescence *in vivo*: telomere length, senescence-associated gene expression, and changes associated with organelles - lipofuscin and senescence-associated β-galactosidase. We studied how these parameters changed with age in three species: humans, rats and mice. We have also begun to analyze human renal age-related changes in gene expression by microarray analysis.

HYPOTHESES: The mechanisms of cellular senescence *in vitro* occur *in vivo* and contribute to the phenotype of renal aging. Measures of senescence will correlate with kidney pathology and function, with the age, comorbidities, injuries, and possible genetic make-up of the individual. Post-transplant immune and non-immune injury accelerates tissue clocks by proliferative or non-proliferative mechanisms, leading to progressive loss of nephrons (allograft nephropathy or AN).

BACKGROUND: Donor age strongly influences late survival of renal transplants due to AN. The pathology of AN overlaps aging, suggesting common elements in their pathogenesis. Both involve loss of nephrons with tubular atrophy, interstitial fibrosis, and fibrous intimal thickening in small arteries. Of the many possible molecular mechanisms in cell/organ aging, basic studies raise replicative senescence as a major possibility. In cultured human fibroblasts replicative senescence is due, at least partially, to telomere loss and increases in negative cell cycle regulators such as p16INK4a. During the course of this project, other laboratories have established that mouse fibroblasts in culture display a senescence-like phenotype with p16INK4a expression, but do not rely on the telomere mechanism. We have obviously incorporated this new knowledge into our project.

SPECIFIC AIMS:

1) To measure telomere length and age-related changes in gene expression in human kidneys.

2) To measure telomere length and age-related changes in gene expression in rat and mouse kidneys and compare these with human kidneys and with the phenotype of kidney aging in these species.

3) To initiate microarray analysis of age changes in gene expression in human kidney, seeking age-specific clusters.

4) To determine whether graft rejection and tissue injury can accelerate the changes of renal senescence.

RESULTS:

A. Telomeres and senescence gene expression in aging human kidney

We established two methods to assess telomere attrition in human kidney specimens: Southern blotting of terminal restriction fragments (TRF); and slot blotting using telomere-specific probes. The results of both methods correlated with each other. The mean TRF length determined by Southern blotting in the cortex was about 12 kb pairs (kbp) in infancy and was shorter in older kidneys (about 8 kbp). The slope of the regression line was 0.029 kbp (0.24%, $p<0.05$) per year. Telomere loss in the ortex by the slot blot method was 0.25% per year ($p<0.05$). Telomere loss in the medulla was not significant and less than in the cortex by both methods (Melk et al. *J Am Soc Nephrol* 2000).

We found that human kidneys from individuals of different ages express relatively constant levels of various mRNAs for genes potentially related to senescence: matrix metalloproteinase 1, plasminogen activator 1, prostaglandin endoperoxidase synthase, transforming growth factor β1, p53, p21CIP1/WAF1, p14ARF, cyclin D CDK4 PCNA, E2F1, suggesting that cell cycle regulation is well maintained over time. However, mRNA levels for p16INK4a rose with age. The cyclin-dependent kinase inhibitor p16INK4a is a mediator of cellular senescence *in vitro*. It plays a key role at the G1 cell cycle checkpoint by inhibition of CDK4 and 6, thereby blocking cell proliferation. p16INK4a mRNA expression was measured using quantitative RT-PCR (TAQMAN-PCR). P16INK4a expression was high in kidneys from older humans. Regression analysis excluding samples with histologic abnormalities revealed a significant increase for cortex, but not for medulla. However, kidneys with abnormal features tended to show high p16INK4a levels even in younger individuals.

Our attempts at staining p16INK4a with commercially available antibodies have been unsuccessful so far because of the unavailability of antibodies that work in tissue sections. Recently, we were able to receive two antibodies from Dr. J. Koh, University of Vermont, that have been shown suitable for tissue sections. The manuscript describing human senescence studies is in preparation, and will be submitted in the next three months.

We assessed the importance of a known *in vivo* marker for senescence: senescence-associated (SA) β-galactosidase (GAL). SA-β-GAL staining was found in all human specimens investigated. Statistical analysis revealed no difference between specimens from young and old individuals. We conclude that if SA-β-Gal is a marker for renal senescence *in vivo*, as it is in skin, then

senescence is occurring at a constant rate in human kidney tissue and is not increased by age. On the other hand, lipofuscin was strongly related to age.

These results are compatible with a model of human renal aging in which nephron loss reflects a response to genomic changes (telomere shortening) which limits replication, and environmental stress which causes "wear-and-tear" organelle deterioration (and lipofuscin), resulting in cell loss. The result is a decline in mass but with relative preservation of cell cycle regulation and gene expression.

B. Senescence mechanisms in rat and mouse kidneys

Rat experiments. Experiments were performed on Fischer 344 rats of three age groups (1, 9 and 24 months). We established the use of pulse field electrophoresis (PFGE) for investigation of telomere length (TRF). Normal rat kidney showed a slight but not significant decrease in TRF of about 3 kbp within two years, but with telomeres being still over 30 kbp long, critical telomere shortening was not reached. This is the first indication that rats do not use telomere shortening as a cell cycle counter, compatible with recent *in vitro* evidence in mouse fibroblasts.

We used TAQMAN PCR to evaluate changes in p16INK4a in rat kidneys. Expression was very low or undetectable in young rat kidneys: only one out of 11 rat kidney samples in the 1-month group had any detectable p16INK4a expression. p16INK4a rose 27-fold in 9-month-old rats and 72-fold in 24-month-old rats. The difference between the three age groups was highly significant. Similar significant increases were seen for the other tissues (brain, heart, spleen) investigated.

Unfortunately, no commercially available antibody is available against rat epitopes of p16INK4a. Using one antibody that is supposed to work in rat tissue, we found positive nuclear staining in all tubular cells regardless of age. Trials to reduce this non-specific staining and leave specific staining have failed, illustrating the inadequacy of reagents for histologic analysis in this area.

Lipofuscin and SA-β-GAL staining in the epithelium of rat kidney specimens increased statistically significant with age. We assessed the pathologic changes of aging in collaboration with Dr. Kim Solez. They included glomerular sclerosis, tubular atrophy and interstitial fibrosis, with no loss of function. At 24 months most nephrons remained histologically normal. The manuscript for rat results has been submitted for publication.

Mouse experiments. We studied BALB/c of four different age groups (newborn, 1, 3 and 18 months). In mouse kidney specimens, mean TRF measured between 40 and 50 kbp and show a small, significant decrease of 2.7 kbp between 1 and 18 months of age. The loss in telomere length was accompanied by a decrease in mTert mRNA expression. However, as in the rat, we can confirm *in vivo* that mice have very long telomeres and do not use telomere shortening as a cell cycle counter *in vivo*, in keeping with the predictions from *in vitro* data.

p16INK4a expression increased progressively over 200-fold, from negligible levels in newborn to high levels in old mice. p16INK4a antibodies stained tubular cells and increased with age. In contrast, p19ARF (the murine correlate for p14ARF) mRNA expression had a more complex

pattern with high expression in newborns and old mice, suggesting its importance for development and senescence. Immunoperoxidase staining for p16INK4a was established for one of two commercially available antibodies and showed nuclear staining of mainly tubular cells. The proportion of nuclei stained increased with age. Unfortunately, we found positive staining to some extent when the same antibody was used to stain kidney tissue derived from a p16INK4a-knockout mouse. SA-β-GAL was found mainly in tubular cells of the juxtamedullary region and increased with age (manuscript in preparation).

C. Microarray analysis of human kidneys for age-related gene clusters

We have performed studies on transcriptional changes using cDNA microarray analysis on human kidney samples of various age in collaboration with Dr. Minnie Sarwal, Stanford University. Initial results show that fewer than 10% of the selected genes showed increased or decreased expression over controls, suggesting that there are a few significant age-specific differences in mRNA levels among human kidneys. The initial data was presented in abstract form at the Washington ATC meeting, April 2002. The results represent the first survey of the effect of age on the expressed genes in a human transplantable organ, and a step toward a molecular understanding of the aging phenotype, and will eventually be published as a separate manuscript. The strategy is to identify individual genes or patterns, and confirm the key observations with real time PCR and if possible by analysis of product expression.

D. Relationship of senescence mechanisms to the renal transplant course

This is the most challenging aspect of the project. In view of the new information that major species differences exist between human and rodent kidneys, we could not proceed with our plan to see if renal injury accelerated telomere shortening in rodent kidneys, since rodents do not use telomere mechanisms.

Since there is a limited amount of tissue available from transplant biopsy specimens, and telomere length requires more DNA than can be obtained in biopsies, we have no data examining the relationship between telomere length and kidney function or performance post-transplant. None of the above used methods proved to be useful. We are developing new techniques to enable us to investigate biopsy specimens. In collaboration with Dr. P.S. Rabinovitch, University of Washington, Seattle, we are currently exploring the use of quantitative fluorescent in situ hybridization to determine cell-specific telomere length. Dr. Rabinovitch and his group were able to use this method on human gut sections derived from patients with ulcerative colitis. In order to measure gene expression, we have established the use of laser capture microdissection and we have already used this technique successfully in other projects.

E. Development of a mouse model to test whether repeated injury will accelerate telomere length attrition and increase senescence gene expression

We studied senescence-associated genes in our mouse transplant models: CBA kidney transplanted into B6 or BALB/c and 129 kidney transplanted into CBA. p16INK4a mRNA expression increased up to 50-fold in some strains after transplantation. p19ARF showed only up to 10-fold changes in kidney transplants compared with the contralateral kidney. Telomerase expression

stayed almost the same. The striking changes in p16INK4a expression in rejecting transplants are previously unreported and are being explored further. SA-β-GAL staining did not change in transplanted kidneys compared with normal controls but showed some differences for the different mouse strains investigated.

Emerging understanding of the senescence phenotype and its relationship to transplantation. The above experiments provide some surprising changes in our understanding of aging and of tissue loss in failing organ grafts, using the kidney as an example. What we are finding is that humans and rodents have different strategies for organ homeostasis, and that studying these systems in parallel can shed light on the general problem of organ homeostasis in age and disease. The general aspects of our emerging model are as follows:

1. Humans use the telomere mechanism as a replication counter – rodents do not. This may explain the preservation of mass in rodent kidneys with age but with a high incidence of cancer. Rat and mouse kidneys simply do not undergo the age-related loss of mass that occurs in human kidneys, do not undergo telomere shortening, but manifest much more striking changes in p16INK4a.
2. Human kidneys lose mass and lose nephrons with age. The mechanism of loss of nephrons is not understood. On the basis of our current results with microarrays, the transcriptosome is well preserved in aged kidneys, suggesting that loss of mass reflects a regulatory mechanism that shuts down cells en masse rather than have them transcriptionally active. Nevertheless, we believe that it will be possible to identify patterns of gene expression ("clusters") characteristic of kidney aging.
3. Human kidneys lose mass and nephrons after transplant injuries – allograft nephropathy. This change simulates renal aging (renal senescence) in an accelerated form, and occurs particularly in kidneys from old donors. Rodents do not develop nephron loss after transplant stresses, but instead rats develop focal and segmental glomerulosclerosis after transplantation. Thus, in rats allograft nephropathy does not occur, but focal sclerosis does. Oddly, transplant stresses and age both produce nephron loss and loss of mass in humans, and focal sclerosis in rats. Thus, both species, despite their many differences, preserve a relationship between transplant stresses and their phenotypic changes of aging.
4. Human, rat, and mouse develop focal changes in the renal epithelium with age resembling senescent cells *in vitro* – lipofuscin staining and SA-β-GAL. Humans also show telomere shortening and lipofuscin but not as strikingly related to age, and show an increase in SA-β-Gal staining.
5. Rodents show very high p16INK4a expression with age. Human kidneys show increased expression of p16INK4a mRNA with age but the increase is more subtle. Other cell cycle regulators change relatively little with age. Staining of p16 is unsatisfactory. Early results in mouse kidney transplants indicate that rejecting mouse transplants express very high levels of p16ink4a, but whether this is a lymphoid or epithelial is under investigation.

It seems likely that this analysis can eventually produce a better understanding of human organ aging that will help in evaluating organs from older donors, and may lead to interventions to preserve their function. We hope that the promising early microarray results will bear fruit, possibly permitting a simplified real-time PCR approach. This could also lead to a mechanistic understanding of the processes involved.

The elusive experiment is to see whether the pathway of nephron loss in transplanted kidneys after injuries (brain death, rejection) is similar to the loss of nephrons with age in normal human kidneys but at an accelerated rate. We now know that the rodent kidney fails to shrink with age or transplant stress, and thus cannot be a reliable model for this event. The data continues to support the idea that nephron loss in response to injury in human kidney transplants (allograft nephropathy) simulates the loss of mass/nephrons in renal aging and reflects common mechanisms, and that the rodent differs fundamentally from the human in these mechanisms and cannot be taken as a model for these chronic changes. Nevertheless, the rodent represents a valuable comparison for developing an understanding of these strategies.

The problem of organ age remains unsolved but the focus of the next generation of investigations should shift to mechanism of shutdown and its regulation. The problem of telomere attrition and cellular senescence is able to be tested by interventions that prevent nephron loss. We have had discussions with a corporate partner - Geron - about drugs to bypass age-related defects, e.g. transient gene therapy with telomerase during periods of stress, e.g. at the time of transplantation. New animal models - the telomerase knockout mouse, with shortened telomeres - may greatly facilitate experimentation in this area. Thus, it seems likely that the new information will lead to the goals.

References and Publications

Halloran PF, Melk A, Barth C. Rethinking chronic allograft nephropathy - the concept of accelerated senescence. *J Am Soc Nephrol* 1999; 10:167-181.

Melk A, Ramassar V, Helms LM, Moore R, Rayner D, Solez K, Halloran PF. Telomere shortening in kidneys with age. *J Am Soc Nephrol* 2000; 11:444-453.

Melk A, Turner P, Helms LMH, Ramassar V, Urmson J, Halloran PF. Expression of senescence regulator p16INK4a mRNA in some human kidney samples. *J Am Soc Nephrol* 2000, 11: S461A (Abstr. 2429).

Melk A, Halloran PF. Cell senescence and its implications for nephrology. *J Am Soc Nephrol* 2001; 12: 385-393.

Melk A, Halloran K, Sandhu I, Gourishankar S, Rayner D, Helms LMH, Halloran PF. Evidence for cellular senescence in tubular epithelium of rodent and human kidneys. *Am J Transplant* 2001; 1:S230 (Abstr. 377).

Melk A, Rayner D, Pehowich ED, Solez K, Halloran PF. Renal senescence reflects different mechanisms in human and rodent kidneys. *J Am Soc Nephrol* 2001; 12: S616A (Abstr. 3207).

Melk A, Sandhu I, Halloran PF. Epithelial cell senecence may contribute to the renal aging phenotype. Abstract presented at ATC 2002.

Melk, Halloran PF, Sarwal M. Gene expression pattern in human renal senescence using microarray technique. Oral presentation at ATC 2002.

Melk A, Kittikowit W, Jhangri GS, Sandhu I, Halloran K, Halloran PF. In vivo expression of cell senescence markers in rat kidneys increases with age. Submitted for publication.

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Effect of Vitamin D on Renal Allograft Function

Our long-term objective is to develop an effective means of preventing chronic rejection (CR) and to identify the mechanisms underlying extracellular cell matrix (ECM) remodeling during CR, in particular chronic allograft nephropathy (CAN). Our data suggest that 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] mediates changes in the extracellular matrix (ECM) that improve/stabilize renal function. This proposal addresses the role of 1,25-$(OH)_2D_3$ in ECM remodeling and may identify new targets for therapy. This objective remains unchanged since the beginning of the project, which was funded for an initial trial period of one year to meet the goals of Specific Aim 1.

We hypothesized that 1,25-$(OH)_2D_3$ may be an effective agent in preventing or limiting CAN. Further, we hypothesize that 1,25-$(OH)_2D_3$ regulates ECM remodeling through an interaction of the VDR with the TGFβ signaling proteins, leading to changes in the expression of proteins responsible for ECM remodeling, namely, the matrix metalloproteinases (MMP) and the tissue inhibitors of MMP (TIMP). In Specific Aim 1 we proposed to determine whether 1,25-$(OH)_2D_3$ treatment is effective in preventing ECM remodeling when used in reduced-dose combination therapy with standard immunosuppressive agents. To accomplish this aim we have chosen to use the DA to Brown Norway model of CR, which develops a strong acute rejection response followed by CAN at 40 to 60 days post-transplant. The table below describes the experimental conditions tested to date.

Recipient	Treatment		
	Cyclosporine A (mg/kg/day)	1,25-$(OH)_2D_3$ (ng/day)	Mean survival time (days)
DA	--	--	60
BN	--	--	6.5 + 0.7
BN	5	--	10.2 + 5.3
BN		100	6.8 + 0.8
BN	5	100	9.7 + 0.9
BN	10		>60
BN	10	100	>60

All recipients treated with 10 mg/kg/day cyclosporine A (CsA) showed no evidence of acute or CAN. We are currently generating a third group of recipients that will be treated with CsA at 7.5 mg/kg/day. Serum calcium levels were elevated in all 1,25-$(OH)_2D_3$ treated groups;

however, no deposits were noted in the transplanted kidney (Figure 1). Urinary protein and serum creatinine levels in the long-term BN recipients were not different that syngeneic control recipients (Figures 2 and 3).

We have also continued analysis of Fisher to Lewis recipients treated with 1,25-$(OH)_2D_3$ as monotherapy. Renal lysates were prepared from Lewis recipients of a Fisher kidney graft placed 24 weeks earlier. Semi-quantitative RT-PCR, immunoblotting and antigen-capture ELISA were used to analyze changes in Smad and MMP expression.

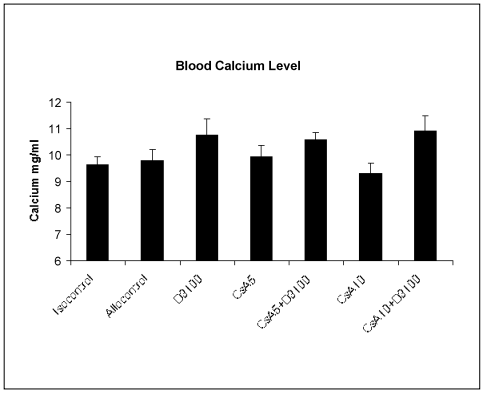

Figure 1. *Serum calcium levels in long-term DA → BN recipients.*

RT-PCR analysis showed no significant change in the expression of Smad 2, Smad 3 or Smad 7 mRNA; however, Smad 6 mRNA expression was significantly increased in allogeneic ($p = 0.02$) and syngeneic ($p = 00.1$) grafts treated with 1,25-$(OH)_2D_3$. Immunoblotting reveal a significant increase in Smad 2 and Smad 6 protein expression in all recipients (5-8 fold change, $p = 0.04$) in comparison with syngeneic untreated controls. Smad 3 expression was not altered in allogeneic recipients regardless of treatment in comparison with syngeneic controls. Smad 7 protein expression increased in allogeneic (5.5-fold, $p = 0.02$) and syngeneic (4-fold) grafts from 1,25-$(OH)_2D_3$-treated recipients and unchanged in untreated allogeneic grafts in comparison with syngeneic untreated grafts.

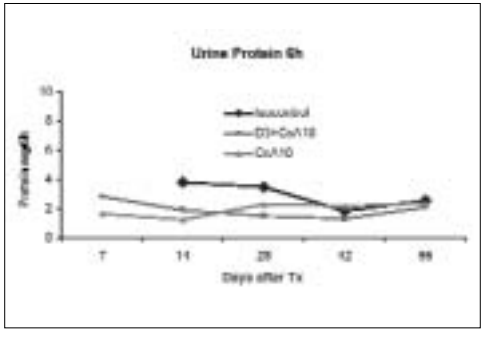

Figure 2. *Urinary protein levels in long-term DA → BN recipients.*

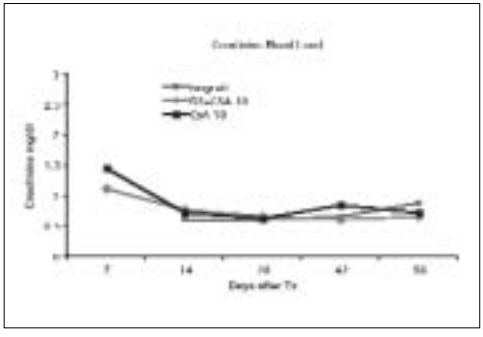

Figure 3. *Serum creatinine levels in long-term DA → BN recipients.*

RT-PCR analysis showed a significant increase in MMP-2 mRNA expression in allogeneic (5.6-fold) and syngeneic (4.0-fold) 1,25-$(OH)_2D_3$ treated grafts, whereas untreated allografts were unchanged (1.3-fold increase) from untreated syngeneic grafts. MMP-9 expression was increased in all groups and was not altered in allogeneic grafts by 1,25-$(OH)_2D_3$ treatment [no treatment 4.0-fold vs 5.7-fold with 1,25-$(OH)_2D_3$].

TIMP-1 mRNA expression was increased in untreated (3.9-fold) and 1,25-$(OH)_2D_3$ treated (3.9-fold) allografts in comparison with untreated syngeneic grafts. 1,25-$(OH)_2D_3$ treatment marginally increased TIMP-1 in 1,25-$(OH)_2D_3$ treated syngeneic grafts (1.5-fold). Antigen-capture ELISA specific for MMP-2 revealed a 2.2-fold decrease in total MMP-2 protein expression in untreated allogeneic recipients. No change was observed in either allogeneic or syngeneic 1,25-$(OH)_2D_3$-treated grafts. Masson's Trichrome staining of paraffin sections showed a significant decrease in collagen deposition in 1,25-$(OH)_2D_3$-treated allografts in comparison with untreated allografts. Finally, 1,25-$(OH)_2D_3$ treatment inhibited the rise in urinary protein that typically occurs at 16-20 days post-transplantation.

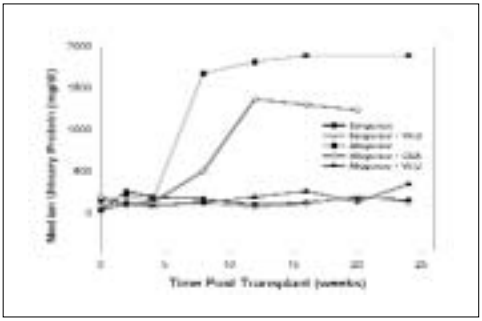

Figure 4. *Urinary protein levels in Fisher → Lewis recipients. Recipients receiving CsA were treated for 10 days at 1.5 mg/kg. 1,25-$(OH)_2D_3$ was administered in the diet until graft harvest.*

In summary, 1,25-$(OH)_2D_3$ treatment of graft recipients increases inhibitory Smad expression, thereby likely preventing TGFβ-1 signaling. Secondly, 1,25-$(OH)_2D_3$ treatment of grafts recipients increases MMP-2 expression, likely accelerating matrix breakdown. In untreated allografts, matrix deposition occurs because the MMP/TIMP ratio favors accumulation. Our data clearly suggest that 1,25-$(OH)_2D_3$ therapy alters the development of CAN by modulating TGFβ-1-regulated gene expression and by altering the expression of proteins that are responsible for ECM remodeling.

Future studies will include identification of a CSA dose that results in CAN and that is prevented by 1,25-$(OH)_2D_3$. We will also pursue combination of 1,25-$(OH)_2D_3$ with mycophenolate mofetil.

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Disordered Thromboregulation in Xenotransplantation

Xenotransplantation, the transplantation of viable cells, organs or tissues between species, has been proposed as a solution to the shortage of human organs for the treatment of organ failure. Although hyperacute rejection (HAR) in experimental discordant combinations can be now effectively managed, vascularized discordant xenografts are still subject to acute vascular rejection (AVR), also referred to as delayed xenograft rejection[1-3]. This latter mode of rejection is associated with vascular-based inflammation associated with xenoreactive antibody deposition, thrombocytopenia and the consumption of coagulation factors that may ultimately evolve to disseminated intravascular coagulation (DIC)[3-5].

The infusion of discordant (porcine) hematopoietic cells in primates to induce by mixed chimerism is also associated with widespread thrombotic vascular injury[6-10]. The mechanisms underlying the consumptive coagulopathy and thrombotic microangiopathy in these settings are unclear. Certainly, antibody-mediated vascular injury and undefined levels of inflammatory mediators or cytokines within the vascularized xenograft could promote vascular thrombosis[11,12]. Molecular incompatibilities can also be shown between primate coagulation factors that would exacerbate this pro-inflammatory and thrombotic situation, e.g. thrombin, and natural anticoagulants, e.g. thrombomodulin (TBM) or factor Xa, and tissue factor (TF) pathway inhibitor (TFPI), on porcine leukocytes and endothelium[12-15].

Our initial approach has been to generate stable xenogeneic cellular transfectants to confirm expression of the required human factor. This has been done successfully for CD39 and TBM; primary porcine and human cultures have been also modified by somatic recombination using viral vectors and functional effects further determined by *in vitro* analyses. Human TFPI is currently being cloned to incorporate a GPI linker, as we have done for von Willebrand factor A1 domains, to boost surface expression[16]. We now plan to develop porcine bone-marrow-derived cell lines over-expressing TBM, TFPI and CD39 following recombinant retroviral infection and to test these for anticoagulant and/or antithrombotic function in baboons.

These above experiments have been conducted in parallel with the generation of mice transgenic for each of the above individual thromboregulatory genes to confirm adequate expression and to check for lethal or deleterious effects, as summarized below.

TFPI: The pcDNA3/TFPI has been cloned into pCEP4 and transiently transfected cells have been used to study the specific anticoagulant activity by inhibition of factor Xa activity in an

indirect assay (TF-dependent pathways)[17, 18]. An additional technique involves inhibition of factor VIIa/TF activity in a direct assay: excess FVIIa (100 pM) is complexed with varying concentrations (0-32 nM) of relipidated human recombinant TF[18]. This work will be supplemented by studies of whole human plasma clotting assays.

Upon testing of transfected cells, minimal TFPI activity has been demonstrated to date, and antibody detection has been compromised by cross-reactivity with TFPI in fetal calf serum. Given that this anticoagulant may be lost from the cell surface with shedding of heparan sulfate, TFPI is currently being cloned with a GPI anchor. We plan to use this GPI-linked TFPI for further experiments, including the microinjections to generate mutant mice transgenic for this human factor. To date, TFPI has been cloned into pEA (H2-K promoter). Microinjections are to be performed and transgenic mice are still pending.

TBM: The cDNA has been cloned into pCMVpuro for transfection. Transfected cells have been incubated with buffer containing calcium and protein C, and thrombin was then added to test activated protein C generation[19, 20]. COS cells were transfected with low efficiency as determined by FACS and anticoagulant properties (measured by the capacity of cell lysates to function as cofactor for protein C). Methods to analyze intact cells with the above assays are in progress. Native TBM has been cloned into pEA (H2-K promoter) for microinjection.

CD39/NTP diphosphohydrolase: cDNA in pcDNA3/CD39 has been cloned into pCMVpuro for transfection into SVAP cells. When transiently transfected into COS cells, CD39 can be detected by FACS with an approximate efficiency of 60%[21]. Substantive increases in activity have been measured by generation of free inorganic phosphate from ADP (or ATP) by transfected vs mock transfected SVAP cells[22,23]. Physiological functions of CD39 will be also determined by inhibition of ADP-mediated platelet aggregation[22,23], as measured in a dual sample platelet aggregometer *in vitro*.

CD39 has been cloned into pEA (H2-Kb promoter) for microinjection, and several litters have been born that over-express human CD39. We have also investigated the utility of the promoter of the Tie2 gene, coding for one of the receptors of the family of regulators of vascular remodeling, called angiopoietins, to facilitate vascular-specific expression. We have obtained plasmid vectors bearing separately the Tie2 promoter (p52) and the long enhancer region (p65) from Dr. Sato (University of Texas, Dallas, USA). We have cut plasmid p52 with HindIII and NotI and ligated the previously purified mCD39 into this plasmid (p52-mCD39). We then digested p52-mCD39 with XhoI and KpnI to remove the short enhancer (responsible for expression of the gene of interest in vascular endothelial cells but only at early stages of embryogenesis) and replaced it with the long enhancer. The long enhancer was isolated from the plasmid p65 following cleavage with NaeI and NotI, purified and blunt-ended. It was ligated into p52-mCD39-XhoI/KpnI, also blunt-ended. New plasmids were checked for the correct orientation of the long enhancer.

The SalI fragment and/or whole plasmid bearing the Tie2 promoter, mCD39 and the long

enhancer has been successfully tested by transient transfection in mouse endothelial cell systems, and used for microinjection; the availability of these transgenic mice is pending (mid-2002). Mice will be screened for this transgene by PCR using primers belonging to two exons to differentiate recombinant mCD39 cDNA from the endogenous genomic CD39.

By generating sufficiently large numbers of founders it will be possible to derive triple transgenics (in which the three genes are co-integrated and inherited en bloc). However, there are obvious caveats in performing the above work. Any experimentation with transgenics to overcome the intravascular coagulation component of AVR in the clinically relevant models will require multi-transgenic pig donors. In the short term, another option would be to make these multi-transgenic coagulation regulator mice and cross them with the multi-transgenic complement regulator/Gal KO mice (to prevent HAR). While we may generate these mice, any *ex vivo* or rodent transplant studies may still have limited application. However, the planned generation and study of transgenic mice remain critical to the process of making transgenic pigs. Firstly, levels of expression obtainable with transfectants are often many fold higher than can be achieved *in vivo* in transgenics, so it must be established that adequate levels can really be achieved *in vivo* and that the expressed molecules are functional (and vice versa). Secondly, these experiments test the promoter *in vivo* in the desired tissue. Third, our plans test for *in vivo* interactions that may occur in triple transgenics; in this case the possibility of a TFPI and TBM interaction is real and may even compromise adequate hemostasis because of potential synergistic activities. Lastly, this strategy tests the exact batch of constructs that will be used later to make the transgenic pigs.

A successful follow-up application to ROTRF will now provide funding over the next 2 years to finalize generation of these mice and further examine defined transplantation models to determine potential utility of this approach in correcting disordered vascular thromboregulation under conditions of inflammatory stress.

References

1. Platt JL, Lin SS, McGregor CGA. *Xenotransplantation* 1998; 5:169-175.
2. Bach FH, Winkler H, Ferran C, Hancock WW, Robson SC. *Immunol Today* 1996; 17:379-384.
3. Bach FH, Robson SC, Ferran C, et al. *Immunol Rev* 1994; 141:5-30.
4. Ierino FL, Kozlowski T, Siegel JB, et al. *Transplantation* 1998; 66:1439-1450.
5. Kozlowski T, Fuchimoto Y, Monroy R, et al. *Transplant Proc* 1997; 29 (1-2).
6. Buhler L, Basker M, Alwayn I, et al. *Transplantation* 2000; 70:1323-1331.
7. Buhler L, Goepfert C, Kitamura H, et al. *Bone Marrow Transplant* 2001; 27:1227-1236.
8. Alwayn IPJ, Buhler L, Appel JZ, et al. *Transplantation* 2001; 71:1601-1609.
9. Appel JZ, Alwayn IPJ, Buhler L, DeAngelis HA, Robson SC, Cooper DKC. *Transplantation* 2001; 72:1299-1305.
10. Appel JZ, Alwayn IPJ, Correa LE, Cooper DKC, Robson SC. *Transplantation* 2001; 72:1306-1310.
11. Buhler L, Yamada K, Kitamura H, et al. *Transplantation* 2001; 72:1743-1752.
12. Sachs DH, Sykes M, Robson SC, Cooper DKC. *Adv Immunol* 2001; 79:129-223.
13. Robson SC. Disordered regulation of coagulation and platelet activation in xenotransplantation. In: Xenotransplantation. Ed. Jeffrey Platt. Humana Press, 2002. In press.

14. Robson SC, Goodman D. Leukocyte adhesion and activation in xenografts. In: Vascular Adhesion Molecules and Inflammation. Ed. Jeremy D. Pearson. Birkhauser Verlag, Basel, 1999.
15. Robson SC, Schulte am Esch II J, Bach F. *Ann NY Acad Sci* 1999; 875:261-276.
16. Schulte am Esch J, Cruz MA, Siegel JB, Anrather J, Robson SC. *Blood* 1997; 90:4425-4437.
17. Kopp CW, Siegel JB, Hancock WW, et al. *Transplantation* 1997; 63:749-758.
18. Kopp CW, Robson SC, Siegel JB, et al. *Thromb Haemost* 1998; 79:529-538.
19. Siegel JB, Grey ST, Lesnikoski BA, et al. *Transplantation* 1997; 64: 88-896.
20. Kopp CW, Grey ST, Siegel JB, et al. *Transplantation* 1998; 66:44-251.
21. Robson SC, Enjyoji K, Goepfert C, et al. *Drug Dev Res* 2001; 53:193-207.
22. Kaczmarek E, Koziak K, Sevigny J, et al. *J Biol Chem* 1996; 271:33116-33122.
23. Robson SC, Kaczmarek E, Siegel JB, et al. *J Exp Med* 1997; 185:153-163.

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The Control of Angiogenesis: Matrix Modulation of Cellular Responses to Cytokines

Background: Angiogenesis (defined as the formation of new blood vessels from pre-existing ones) is an integral feature of many physiological and pathological processes, including trophoblast implantation, wound healing and cancer progression. Angiogenesis, and the resultant establishment of an adequate blood supply, is also a fundamental requirement for the successful integration of tissue engineered grafts and the survival of organ transplants.

Angiogenesis is a multi-step process involving several cell types, including endothelial cells, pericytes and fibroblasts. Cell migration and endothelial sprout formation are key events of the angiogenic process and may be studied *in vitro* using two quantitative assays developed in our laboratory [Schor et al, 2001a,b].

The nature of the macromolecular matrix has previously been reported to exert a significant modulatory effect on the response of target cells to motogenic cytokines and soluble angiogenic factors [Kondo et al, 1993; Schor, 1994]. Our own results indicate that the macromolecular matrix may be either permissive or non-permissive with respect to such factors [Canfield and Schor 1995; Schor et al, 1999a].

Migration Stimulating Factor (MSF) is a novel 70-kDa protein identified and cloned in our laboratory (EMBL accession number AJ276395). It is the first (and so far only) truncated isoform of fibronectin to be identified, consisting of the fibronectin amino terminal fibrin- and gelatin-binding domains. MSF is constitutively produced by foetal skin fibroblasts, but not their adult counterparts. Purified MSF has been reported to stimulate both migration and hyaluronan (HA) synthesis by target adult cells, with half-maximal activity manifest at femtomolar concentrations. [Schor et al, 1989]. These bioactivities of MSF are mimicked by its constituent gelatin-binding domain (GBD) [Schor et al, 1996], and by synthetic peptides containing the IGD amino acid motif, as present in the seventh and ninth type I modules of GBD [Schor et al, 1999b]. It should be noted that these potent bioactivities are cryptic (ie. not expressed) in all known full-length fibronectin isoforms. MSF has been detected in wound fluids collected from actively healing surgical wounds [Picardo et al, 1992]. More recently, MSF, GBD and IGD peptides have been demonstrated to induce angiogenesis *in vivo* [unpublished data].

Objectives: The specific aims of the proposed study have been concerned with elucidating the role of the extracellular matrix in modulating the functional response of endothelial cells, pericytes and fibroblasts to MSF *in vitro*.

Results obtained during the tenure of this grant may be summarised as follows:

1) *The assessment of chemotaxis and chemokinesis in different in vitro assays*

Chemotaxis and chemokinesis are commonly studied using the transmembrane (or "Boyden chamber") assay. Considering the important modulatory role played by the extracellular matrix in defining cell motogenic response, we have developed a quantitative "sandwich" assay which allows a distinction to be made between chemokinesis and chemotaxis within a biologically relevant 3-dimensional matrix [Schor et al, 2001b]. In this assay, cells are plated on the surface of a 3-dimensional native type I collagen gel; thirty minutes after plating (allowing sufficient time for the cells to attach to the collagen fibres, but not to spread or induce fibre re-organisation) this substratum is overlaid with a second gel. As a result of this procedure, the plated cells become embedded within an isotropic 3D collagenous matrix. The gel sandwich is then overlaid with growth medium. The subsequent movement of individual cells (both up or down from their original location) is assessed microscopically at various times thereafter. The angiogenic factor to be tested may be added in equal concentration to both gel layers and the medium compartment in order to ascertain its chemokinetic activity; conversely, it may only be added to the medium compartment in order to establish a concentration gradient within the gel and thereby test its chemotactic activity (Figure 1).

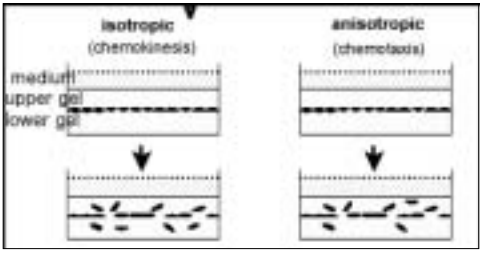

Figure 1. *Sandwich Assay Protocol. Shaded side bars represent the distribution of soluble motogenic factor at the beginning (upper) and end (lower) of the assay.*

A comparison of results obtained using the transmembrane and the sandwich assays has demonstrated that:

(a) The migration of both fibroblasts and endothelial cells were stimulated by MSF, GBD and IGD peptides in both assays, using a native type I collagen substratum. Differences between these target cells were observed regarding dose response and kinetics of migration. In both cell types, manifestation of MSF activity was dependent on the maintenance of integrin $\alpha_v\beta_3$ functionality.

(b) MSF, GBD and IGD peptides stimulated both chemokinesis and chemotaxis of endothelial cells and fibroblasts. Other angiogenic factors have previously been reported to stimulate only chemokinesis.

(c) MSF also stimulated pericyte migration (only chemotaxis tested).

(d) Other angiogenic factors were examined. Some of these were active in both assays (e.g. FGF-2, TGF-β_3), whilst others (eg. TGF-β_1) were only active in the transmembrane assay.

(e) The difference in migration-stimulating activity of TGF-β isoforms in the sandwich assay is consistent with their distinct effects on wound healing *in vivo*.

2) MSF bioactivities and modulation by the extracellular matrix

MSF bioactivity has been examined in terms of the induction of endothelial cell sprouting, proliferation and migration.

(a) *Endothelial cell sprouting.* During angiogenesis, activated endothelial cells migrate through the vessel basement membrane into the surrounding connective tissue stroma, where they adopt an elongated sprouting cell phenotype [Ishibashi et al 1987]. This involves the translocation of endothelial cells from the 2-dimensional luminal environment into a 3-dimensional macromolecular matrix. We have developed a tissue culture assay which mimics early stages of the angiogenic response [Schor et al, 1999a, 2001a]. In this protocol, endothelial cells are plated on a 2-dimensional substratum (eg. tissue culture dish or surface of a collagen gel) where they proliferate and form a confluent monolayer resembling the resting endothelial cells lining the vessel lumen. In the absence of exogenously applied angiogenic factors such cultures maintain this quiescent state indefinitely. The addition of an angiogenic factor results in the appearance of a sprouting cell network within and below the subendothelial matrix. Sprouting cell density may be quantified by standard morphometric analysis. Using this assay, we found that endothelial cells (both large vessel and microvessel-derived) are induced to form sprouts by MSF, GBD and IGD peptides. This effect occurred irrespective of whether the cells were plated on a native collagen or a denatured collagen substratum.

(b) *Cell proliferation.* MSF did not affect the proliferation of endothelial cells, pericytes or fibroblasts under various conditions tested, including the use of different extracellular matrix molecules as substratum.

(c) *Cell migration.* In the transmembrane assay, MSF, GBD and IGD were all found to stimulate fibroblast migration using membranes coated with native type I collagen, but were devoid of motogenic activity when the cells were adherent to membranes coated with denatured type I collagen [Schor et al, 1996, 1999b; unpublished data]. In contrast, endothelial cells responded to MSF, GBD and IGD when adherent to either native or denatured type I collagen, although the response was more marked on the former. Endothelial cells also responded to MSF when adherent to a variety of other matrices tested, including cellular fibronectin, plasma fibronectin and type IV collagen. However thrombospondin-1 (TSP-1) and hyluronan (HA) were non-permissive for the motogenic effects of MSF.

The effects of TSP-1 and HA were further investigated by using these molecules mixed with native type I collagen in various proportions (from 10 to 90%). The effects of TSP-1/collagen and HA/collagen mixtures were investigated in terms of cell attachment, as well of cell migration for both endothelial cells and fibroblasts. Even low concentrations of TSP-1 and HA rendered the collagenous matrices non-permissive for MSF without affecting cell attachment. In parallel experiments, TSP-1 and HA were permissive for the motogenic effect of PDGF and serum. Both TSP-1 (further investigated in a parallel project) and HA (see below) had different effects when presented to the cells in a soluble form.

3) *The role of HA in cell migration.*

The effects of HA are of particular interest, as MSF stimulates fibroblasts to synthesise high molecular weight HA, which is secreted in a soluble form in the culture medium [Schor et al, 1989]. We therefore investigated the effects of soluble HA on cell migration. When presented to cells in this form, HA (both low and high molecular weight) stimulated the migration of endothelial cells and fibroblasts in the transmembrane assay. Fibroblast migration was also examined using 3-D collagen gels. In this assay, soluble low and high molecular weight HA also stimulated cell migration, whereas octasaccharide fragments had no effect. Soluble HA-stimulated migration was mediated by the HA receptor CD44. The above experiments were performed using two lines of human adult-derived fibroblasts. A comparison with foetal-derived fibroblasts (three lines) revealed significant differences. For example, (i) when tested under identical conditions, foetal fibroblasts secrete higher levels of HA and migrate into collagen gels to a greater extent than adult fibroblasts [Chen et al, 1989]. The high levels of foetal fibroblast migration can be reduced to adult-like levels by incubation with either hyaluronate lyase or antibodies to CD44. (ii) Foetal fibroblast migration was inhibited by soluble HA (low and high molecular weight, as well as octasaccharide fragments).

Conclusions: MSF and related compounds induce endothelial sprouting and stimulate the migration of endothelial cells, pericytes and fibroblasts. MSF has no effect on cell proliferation. The motogenic effects of MSF are matrix-dependent for both endothelial cells and fibroblasts. Extracellular matrix molecules may have very different effects depending on whether they are presented to the cells in a soluble form or as an insoluble substratum. HA secretion and deposition may be part of the regulatory mechanism that controls MSF activity *in vivo*.

Our results highlight the important role of the extracellular matrix, as well as cell heterogeneity, when choosing experimental conditions *in vitro*.

Efforts to induce angiogenesis *in vivo* must be carefully balanced by inhibitory signals in order to prevent excessive vessel growth and its associated pathological sequellae. The data produced by this study provide a novel insight into the manner by which this balance may be achieved by the critical interplay of soluble angiogenic factors, and both soluble and insoluble components of the macromolecular matrix.

Our results support the view that MSF and IGD synthetic peptides might prove clinically efficacious in promoting host acceptance of transplanted organs and tissue engineered organ replacements by stimulating the ingrowth of new blood vessels. This conclusion is in keeping with recently obtained data indicating that these compounds do indeed significantly stimulate fibroblast and vessel ingrowth into implanted acellular dermal grafts *in vivo*.

References

Canfield AE, Schor AM. Evidence that tenascin and thrombospondin-1 modulate sprouting of endothelial cells. *J Cell Sci* 1995;108:797-809.

Chen J, Grant ME, Schor AM, Schor SL. Differences between adult and foetal fibroblasts in the regulation of hyaluronate synthesis: correlation with migratory activity. *J Cell Sci* 1989; 94:577-589.

Ishibashi T, Miller H, Orr G, Sorgente N, Ryan SJ. Morphologic observation on experimental subretinal neovascularization in the monkey. *Invest Ophthalmol Vis Sci* 1987; 28:1116-1130.

Kondo H, Matsuda R, Yonezawa Y. Autonomous migration of human fetal skin fibroblasts into denuded area in a cell monolayer is mediated by basic fibroblast growth factor and collagen. *In Vitro Cell Dev Biol Anim* 1993; 29A:929-935.

Picardo M, McGurk M, Schor, SL. Identification of migration stimulating factor in wound fluid. *Exp Mol Path* 1992; 57:8-21.

Schor AM, Ellis I, Schor SL. Collagen gel assay for angiogenesis. Induction of endothelial cell sprouting. In: *Methods in Molecular Medicine*, Vol 46: Angiogenesis Protocols. (ed JC Murray). Humana Press Inc., Totowa, NJ, 2001, pp. 145-162.

Schor AM, Ellis I, Schor SL. Chemotaxis and chemokinesis in 3D macromolecular matrices. Relevance to angiogenesis. In: *Methods in Molecular Medicine*, Vol 46: Angiogenesis Protocols (ed JC Murray). Humana Press Inc., Totowa, NJ, 2001, pp. 163-184.

Schor AM, Schor SL, Baillie R. Angiogenesis: Experimental data relevant to theoretical analysis. In: On Growth and Form: Spatio-temporal Patterning in Biology (ed MAJ Chaplain, GD Singh, J McLachlan). John Wiley and Sons, 1999, pp. 201-224.

Schor SL. Cytokine control of cell motility: modulation and mediation by the extracellular matrix. *Prog Growth Factor Res* 1994; 5:223-248.

Schor SL, Ellis I, Banyard J, Schor AM. Motogenic activity of the IGD amino acid motif. *J Cell Sci* 1998; 112:3879-3888.

Schor SL, Ellis I, Dolman C, Banyard J, Humphries MJ, Mosher DF, Grey AM, Mould AP, Sotille J, Schor AM. (1996) Substratum-dependent stimulation of fibroblast migration by the gelatin-binding domain of fibronectin. *J Cell Sci* 1996; 109:2581-2590.

Schor SL, Schor AM, Grey AM, Chen J, Rushton G, Grant ME, Ellis I. Mechanism of action of the migration stimulating factor (MSF) produced by fetal and cancer patient fibroblasts: effect on hyaluronic acid synthesis. *In Vitro* 1989; 25:737-746.

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Integrated TCR Vβ Transcriptome Analysis, a New Method to Follow Allo- or Auto-Immune Responses

Using our new global method of T cell repertoire analysis referred to as TcLandscape, we showed that purified naive T cells confronted *in vitro* with allogeneic antigen presenting cells (APC) in a direct pathway-restricted MLR up-regulate their Vβ mRNAs without exhibiting skewing of Complementarity Determining Region 3 (CDR3) length distribution. This observation was described in an article published in *Journal of Immunology* (Sebille et al. *J Immunol* 2001; 167:3082-3088). In another article (Guillet et al. *J Immunol* 2002; 168:5088-5095) using the same approach, we showed *in vivo* that Vβ transcript regulation and CDR3 length distribution follow the same pattern during acute rejection of MHC incompatible heart allografts. In contrast, in tolerance induction by priming of recipients with donor cells, the vigorous Vβ mRNA accumulation with Gaussian CDR3 length distribution is abolished, providing a possible explanation for the down-regulation of activated T cells in tolerant animals. In addition, tolerated grafts harbour T cells with a highly altered repertoire suggestive of self-restricted presentation with some patterns corresponding to previously identified regulatory cells.

Moreover, we have previously shown that in the concordant hamster-to-rat cardiac xenograft model, recipients treated with Cobra Venom Factor (CVF) in the first ten days following transplantation and daily with Cyclosporine A (CsA) do not reject their graft. However, a cellular acute rejection is obtained within 4 days when CsA is withdrawn on day 40. Allografts performed in the same recipient strain receiving the same immunosuppressive regimen reject their graft in 18 days following CsA interruption. In these models, we analysed T cell mobilisation through our TcLandscape approach. We showed that in the first days following transplantation, under CsA therapy, TCR accumulation was weaker in allografts than in xenografts. In addition, xenograft acute rejection following withdrawal of CsA involved a high recruitment of activated T cells without skewing of their CDR3 length distribution. In contrast, T cells infiltrating allografts at rejection time have a highly altered repertoire. Interestingly, some Vβ families highly accumulated in rejected allografts with strongly altered transcripts were also found accumulated in rejected xenografts but without CDR3 skewing. Using a correspondence factor analysis, we also found that, in terms of Vβ transcript accumulation, some families cluster (and discriminate) with the allo- or xeno-pattern whereas others were similarly mobilised in both situations, despite the strong difference in the stimulating antigens. The polyclonal pattern observed during xenograft rejection could

be due to the involvement of a direct-type pathway of recognition, which also occurs without CDR3 skewing, or to the presentation of a large variety and quantity of xeno-peptides through the self-restricted MHC pathway (or both). In contrast, the altered profile of accumulated Vβ families observed in allografts at rejection time is concordant with the recognition of peptides presented in the context of the self-MHC. Finally, qualitative analysis of the alterations of the CDR3 length distribution profiles observed in allo- and xeno-situations is characterised by a same global Gaussian CDR3 length distribution when assessed at the level of the whole repertoire of each individual. This suggests for the first time an active general process of homeostatic T cell regulation, where down-regulation of T cell clonal populations is compensated by over-regulation of other ones within a same individual. These observations are detailed in an article submitted for publication.

In addition, during the past year, the technique has been improved by designing a new set of specific primers in rats, mice and humans. Moreover, we have now a 393 wells TaqMan fed by a robot and the CDR3 sizes are directly studied on a capillary sequencer from amplified products. The amount of mRNA needed for the entire process has been decreased from about 10 to 1 microgram.

Publications

Sebille F, Gagne K, Guillet M, Degauque N, Pallier A, Brouard S, Vanhove B, Delsuc MA, Soulillou JP. Direct recognition of foreign MHC determinants by naive T cells mobilises specific Vβ families without skewing of the complementarity-determining region 3 length distribution. *J Immunol* 2001; 167:3082-3088.

Guillet M Brouard S, Gagne K, Sebille F, Cuturi MC, Delsuc MA, Soulillou JP. Different qualitative and quantitative regulation of Vβ TCR transcripts during early acute allograft rejection and tolerance induction. *J Immunol* 2002; 168:5088-5095.

Guillet M, Sebille F, Soulillou JP. TCR usage in naive and committed alloreactive cells: implications for the understanding of TCR biases in transplantation. *Current Opin Immunol* 2001; 13:566-571.

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The Role of Chronic Tissue Hypoxia in the Pathogenesis of Chronic Allograft Rejection

The role of hypoxia in transplantation and its likely responsibility in the development of chronic rejection has not been fully realised. Tissue hypoxia occurs at various times in the course of transplantation, during the procurement of the graft, during the cold storage period and during the surgery to implant the graft. Additionally, a hypoxic microenvironment may exist within the vessel wall as a result of intimal or medial cell proliferation resulting in O_2 diffusion impairments. Taken together hypoxia can be an initial insult as well as a chronic stimulus, which could be a key in the development of chronic rejection.

The aim of our work is to establish that chronic tissue hypoxia, as it may evolve during the early vascular remodelling process as a direct consequence of progressive vessel thickening, is an important allo-antigen independent factor in the process of chronic rejection. The presence of hypoxia-responsive genes, such as PDGF, IL-8, IL-6 and LFA-1, in chronically rejected grafts supports the hypothesis that chronic tissue hypoxia acts as an enhancer of inflammatory responses and is an important contributor to the development and irreversibility of chronic vascular and parenchymal cell rejection. To investigate the role of hypoxia in chronic rejection we are focusing on the activity of a hypoxia inducible transcription factor, HIF-1, that has already been implicated as a critical factor in the pathogenesis of tumour vascularisation, myocardial ischemia and stroke by having a regulatory role in localised tissue hypoxia prevailing in these conditions.

In the first year of this study we began to determine the severity and occurrence of tissue hypoxia during transplantation. In our initial experiments we used established animal transplant models and have demonstrated by IHC that native mouse and rat hearts have localised expression of HIF-1, namely in cardiomyocytes, smooth muscle cells (SMC), endothelial cells (EC), and weak staining of interstitial macrophages. After 24 hours, transplanted grafts had focal areas of sub-endocardial necrosis associated with a decrease of HIF-1 in the cardiomyocytes and in the vasculature. Interestingly, HIF-1 expression increased in the interstitial macrophages whilst infiltrating neutrophils were negative. We are continuing to analyse heart tissue post operative day 30 and 60, with preliminary data suggesting an increase of infiltrating macrophages and an increase in ET-1 and VEGF in these grafts compared to native ones.

For a more specific marker of hypoxia we are constructing a transgenic mouse carrying a hypoxia responsive reporter gene. This transgenic line will ultimately be used in a cardiac transplant model to study the kinetics and pattern of intragraft tissue hypoxia in correlation

with the process of chronic rejection. To date we have constructed a hypoxia responsive reporter gene with a 50-fold induction of β-galactosidase activity under hypoxia, as well transgenic Balb/c lines that have neuronal expression of the reporter gene. We are continuing to screen additional transgenic lines and are using *in vitro* methods of differentiating ES cells, as well as generating teratocarcinomas in nude mice in order to locate reporter activity in differentiated tissue.

The second aim our initial proposal was to investigate, *in vitro*, the interaction of cell types involved in the development of chronic allograft rejection in the context of hypoxia-driven cellular responses. Focusing first on the role of the SMC, we have successfully isolated human primary SMC from aortic and arterial branches and have cultured them both in a secretory (synthetic) and contractile phenotype. Serially passaged, explanted SMC have a phenotype that is consistent with the secretory SMC found in the intimal thickenings of atherosclerotic plaque. For example these cells are capable of rapid replication, are migratory and secrete inflammatory chemokines phenotypes. Explanted SMC can be transformed into a phenotype consistent with the contractile SMC of the healthy medial layer of the artery by altering the *in vitro* culture conditions (i.e., removal of growth factors and reduction of serum). Using immunoassays and reverse transcriptase polymerase chain reaction (RTPCR) SMC were screened in both phenotypes for the expression of inflammatory cytokines and chemokines as well as SMC marker proteins such SMCα-actin. A phenotyping protocol based on the presence or absence of IL-8, MCP-1 and levels of expression and cytoskeletal organisation of SMCα-actin is now routinely used to differentiate the SMC phenotypes.

Both phenotypes respond to hypoxia (2% O_2) by increasing the nuclear accumulation of HIF-1α. Hypoxia is also increasing the transcriptional activation of IL-1α, FGF and VEGF in these cells. Based on the hypothesis that chronic hypoxia results in SMC proliferation, extracellular matrix accumulation and vessel wall remodelling, we are continuing these studies in order to determine whether this is a direct effect of hypoxia on the SMC, or indirect, through an increase of vascular mitogens produced by the endothelium, i.e., PDGFβ, ET-1, VEGF. Additionally we have investigated the role of hypoxia on SMC proliferation, specifically by modulating cell cycle progression through the regulation of cell cycle regulatory proteins and mitotic inhibitors, i.e. p27. Interestingly, hypoxia has a proliferative effect on SMC and we observed that the expression of the cell cycle inhibitory protein p27 decreased under hypoxia (Figure 1). This is contrary to what was recently published, in which fibroblasts respond to hypoxia by increasing p27 protein. It appears that cells of the vasculature may have different proliferative responses to hypoxia, one mechanism via the expression of p27. As a result of hypoxia, fibroblasts increase p27, which in turn decreases cell proliferation and SMC decrease p27 thus allowing SMC proliferation to proceed.

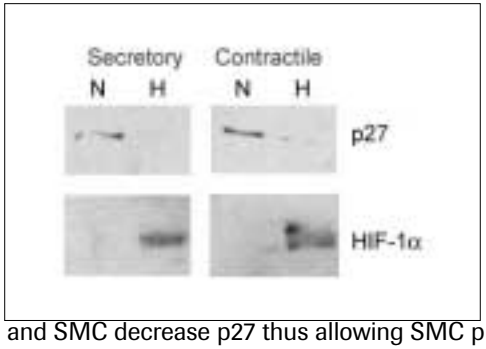

Figure 1. *Immunoblot for HIF-1α and p27 protein.*

This novel observation provides additional

proof for the role of hypoxia in SMC proliferation, and indirectly gives evidence that hypoxia is an enhancing factor in the progression of neo-intimal proliferation, characterised by SMC proliferation and migration.

In summary, this year's work has provided us with preliminary data for our investigations of the importance of hypoxia in transplantation. Together with animal models, we will continue with a genomic analysis of hypoxic SMC and hope to reveal novel patterns of gene expression which could contribute to the initiation of neo-intimal proliferation and promotion of inflammation within the artery wall which leads to chronic graft rejection. This information will also significantly contribute to later studies in which we will investigate the ability of SMC exposed to hypoxia to regulate the recruitment of flowing leukocytes to endothelial cells. To establish the correlation between hypoxia-dependent gene regulation and development of chronic rejection may be of importance for defining novel molecular targets for therapeutic interventions.

Part of this work was presented as an oral presentation at the Keystone Symposia, Inflammatory Paradigms and the Vasculature II. Steamboat Springs Colorado, April 9–14.

Stroka DM and Candinas D (2002) HIF-1 Mediated Responses to Hypoxia and Inflammation in the Vasculature.

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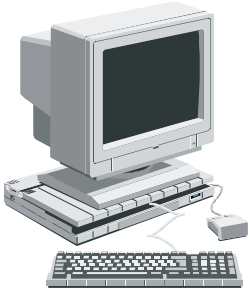

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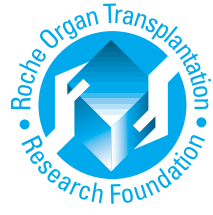

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