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Platelet-derived growth factor in acute and chronic renal allograft rejection

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Academic Dissertation

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ORIGINAL PUBLICATIONS

This thesis is based on the following original publications referred to in the text by their Roman numerals and unpublished data presented in the results.

I Savikko J, Kallio EA, von Willebrand E. Early induction of platelet-derived growth factor ligands and receptors in acute rat renal allograft rejection. *Transplantation* 2001; 72: 31-37

II Savikko J, Kallio EA, Taskinen E, von Willebrand E. The effect of acute rejection and CsA-treatment on induction of PDGF and its receptors in the development of chronic rat renal allograft rejection. *Transplantation* 2002; 73: 506-511

III Savikko J, Taskinen E, von Willebrand E. Chronic allograft nephropathy is prevented by inhibition of PDGF receptor: Tyrosine kinase inhibitors as a potential therapy, *Transplantation* 2003; 75: 1147-1153

IV Savikko J, Teppo A-M, Taskinen E, von Willebrand E. Different effects of tacrolimus and cyclosporine on chronic rat renal allograft nephropathy and PDGF induction: evidence for improved allograft survival, *submitted*

ABBREVIATIONS

APC	antigen presenting cell
AZA	azathioprine
CADI	chronic allograft damage index
CMV	cytomegalovirus
CsA	cyclosporine A
CTL	cytotoxic T lymphocyte
DA	Dark Agouti rat strain
ELISA	enzyme-linked immunoassay
FGF	fibroblast growth factor
FK506	tacrolimus
FKBP	FK506 binding protein
GAP	GTPase-activating protein
HLA	human leukocyte antigen
IFN-γ	interferon gamma
i.p.	intraperitoneally
IGF	insulin-like growth factor
IL-2	interleukin-2
IL-2R	interleukin-2 receptor
LFM	leflunomide
MEIA	microparticle enzyme immunoassay
MHC	major histocombatibilty complex
MMF	mycophenolate mofetil
6-MP	6 -mercaptopurine
NK cell	natural killer cell
NO	nitric oxide
PCR	polymerase chain reaction
PAP	PDGF-associated protein
PI-3 kinase	phosphatidylinositol-3'-kinase
PLC-γ	phospholipase C- γ
p.o.	perorally
PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
RIA	radioimmunoassay
RPM	rapamycin
RT	room temperature
RTK	receptor tyrosine kinase
s.c.	subcutaneously
SMC	smooth muscle cell
SSV	simian sarcoma virus
Tac	tacrolimus
TCR	T-cell receptor
TGF-β	transforming growth factor-β
Th cell	helper T-cell
TNF	tumor necrosis factor
TPA	phorbol ester 12-O-tetradecanoyl-phorbol-13-acetate
VEGF	vascular endothelial growth factor
WF	Wistar Furth rat strain

INTRODUCTION

First succesful human kidney transplantation was done in 1954 between identical twins. In the early days of kidney transplantation grafts were lost shortly after transplantation mainly to acute rejection. A major breakthrough in the treatment of acute rejection was the introduction of cyclosporine in the late 70's. Although Cyclosporine has improved the short-term results it has failed to improve the long-term results, the annual rate of graft loss after the first year and the half-life of transplants have only slightly improved (Paul and Fellström. 1992, Cecka 1999).

Chronic rejection or chronic allograft nephropathy, the term preferred today, is still the major reason for late allograft loss in clinical kidney transplantation. Chronic allograft nephropathy is an irreversible fibrotizing process leading eventually to the loss of the graft, currently there is no treatment available for preventing it. Nowadays it is known that the development of chronic allograft nephropathy is a multifactorial process including both immunological and nonimmunologic factors (Halloran et al. 1999, Paul 2000). However, the exact mechanisms leading to chronic allograft nephropathy are largely unknown.

Although most kidneys survive well after transplantation with modern immunosuppressive medication, acute vascular rejection is still a significant clinical problem early after transplantation. Affected kidney grafts are usually lost, because this type of rejection is often resistant to immunosuppressive medication, it is also called steroid-resistant rejection.

Both in acute vascular rejection and chronic allograft nephropathy tissue macrophages and monocytes that circulate into tissues from blood have an important role (von Willebrand et al. 1992, Croker et al. 1996). They are the major celltypes synthesizing growth factors. Platelet-derived growth factor (PDGF) is one of the most ubiquitous of these peptide regulatory growth factors. PDGF is suggested to be a major mesenchymal mitogen in the development of chronic allograft nephropathy (Fellström et al. 1989, Alpers et al. 1996, Floege et al. 1998). However, its definite role and importance in the rejection mechanisms is unknown but it can be significant both in acute rejection as a mediator which starts the rejection process and also in chronic allograft nephropathy as a mediator that regulates the inflammatory cascades leading to fibrosis and transplant arteriosclerosis.

The aim of this study was to investigate the role of PDGF in acute and chronic renal allograft rejection, and to study the molecular mechanisms between acute rejection and subsequent development of chronic allograft nephropathy as well as to study the long-term effects of new immunosuppressive drugs on chronic allograft nephropathy and PDGF expression.

REVIEW OF THE LITERATURE

1. Clinical kidney transplantation

1.1. History

The first successful experimental kidney transplantation was reported by Emerich Ullmann in 1902 in Vienna, Austria, where he performed a kidney transplantation to a dog. The rapid advance in experimental and clinical surgical skills and the interest of many pioneering surgeons in vascular surgical techniques were the reasons for the interest in transplantation in the early part of the last century. The early experiments simply established that kidney transplantations were technically possible, although allografts eventually failed after functioning briefly. The uncertainty of mechanisms of allograft rejection together with the fact that accurate studies of transplant function were impossible one hundred years ago led to a diminished interest in organ transplantation after some years of activity (Hamilton, 1988).

In the early 1950's, there was renewed interest in experimental and clinical kidney transplantation (Hamilton 1988). Based on experimental transplantations there was a growing certainty that immunological mechanisms were involved in kidney allograft destruction after transplantation Simonsen 1953, Dempster 1953).

The modern and continuing era of transplantation began in the late 1950's, when the first succesful human kidney transplantation was performed between indentical twins in 1954 (Murray et al. 1958). The first attempts at immunosuppression for organ transplants utilized total body irradiation (Murray et al. 1960). Results with total body irradiation showed a high mortality rate due to excessive infectious complications. 6-mercaptopurine and prednisone were used as the first successful chemical immunosuppression in early 1960's (Kuss et al. 1962). Soon the regular use of prednisone and azathioprine became a standard regimen for immunosuppression for the next two decades (Starzl et al. 1963). Antithymocyte and antilymphocyte globulins were introduced during the 60's, and were soon used also routinely to prevent acute rejection (Hamilton 1988). The improvements in the knowledge of allograft rejection and tissue typing led also to a better survival early after kidney transplantation (Hamburger et al. 1962, Ting and Morris 1978).

A real breakthrough in kidney transplantation was the introduction of cyclosporine (CsA) in the late 70's (Calne et al. 1978). CsA therapy revolutinized clinical organ transplantation. CsA dramatically decreased the incidence of acute rejection, and prolonged the early survival of kidney transplants.

However, the long-term survival of kidney transplants has not improved in the CsA era (Paul and Fellström 1992, Cecka 1999).

During the 1990's three new potential agents were introduced for transplant maintenance immunosuppression: mycophenolate mofetil (MMF), tacrolimus (Tac) and sirolimus. Currently Tac is used successfully as a de novo agent for acute rejection prophylaxis and for rescue therapy in kidney transplantation. Mycophenolate mofetil and sirolimus are also used to prevent kidney allograft rejection. The acute rejection episodes have decreased using these new immunosuppressants compared to CsA (Margreiter 2002, Sollinger 1995, MacDonald 2001). However, the effects of these new drugs on long-term kidney allograft outcome are to be seen.

1.2. Indications

Today kidney transplantation is the treatment of choice for patients with end-stage renal failure as a result of improved patient and graft survival. However, only a minority of those patients can eventually be transplanted because of various medical contraindications. The most common indications for kidney transplantation are diabetic nephropathy, chronic glomerulonephritis, cystic renal diseases, nephroscelorosis and amyloidosis. In Finland approximately 150-200 kidney transplantations are performed annually, diabetic nephropathy is the most common indication for transplantation (Salmela and Kyllönen, 2003).

1.3. Kidney allograft rejection

Invasion of the body by any foreign material leads to activation of the immune system. This includes both a nonspesific inflammatory and an antigen-spesific immune response. The spesific immune response is mediated by T-cells and the inflammatory response is mediated by a variety of cells including macrophages, polymorphonuclear cells and NK cells. Rejection is defined as an immune response that induces and mediates injury and destruction in the allograft. It has proved to be the major barrier to transplantation. Rejection has been defined in three categories in clinical transplantation: hyperacute or accelerated rejection, acute rejection and chronic rejection (Dallman and Morris 1988).

Hyperacute rejction occurs immediately on the re-anastomosis or in the first 48 hours of transplantation. This type of rejection is mediated by immune mechanisms that have been activated by exposure to alloantigen prior to transplantation. Hyperacute rejection is clinically a rare cause of graft loss because it can be avoided by adequate antibody cross-match and blood group match.

Acute rejection occurs at the earliest several days after transplantation and most frequently in the first three months. It is a result of a primary response to the graft after graft implantation and can attack all cells in the graft. Acute rejection used to be the most common reason for graft loss, but now with modern immunosuppressive medication it causes less than 10% of the graft losses in the first post-transplant year (Hariharan et al. 2000). Most of these acute rejections, which result in graft loss, are histologically classified as acute vascular rejections.

Chronic rejection can occur any time after the first months of kidney transplantation. It is a slowly ongoing process leading eventually to the loss of the graft. It affects usually vasculature and other graft structures, histopathological findings of chronic rejection are classical for each organ. Clinically chronic rejection is the major unsolved problem in transplantation, as it is less responsive to current immunosuppressive therapies.

The clinical diagnosis of kidney transplant rejection is based on clinical evidence of graft dysfunction, tests identifying systemic activation of the immune system and examination of graft tissue for evidence of inflammation and tissue injury. The primary means by which the renal allograft function is monitored during posttransplant periods is by serial determinations of the serum creatinine level. An approximately 20% elevation in serum creatinine above baseline values signals the need for further evaluation. The standard for determining acute rejection is the renal allograft biopsy. Biopsies are now routinely performed using real-time ultrasound guidance and small-gauge automated biopsy devices (Mendelssohn and Cole 1995). Interpretation of renal biopsy specimens for diagnosing rejection has been greatly facilitated and standardized using the Banff criteria for renal transplant rejection (Solez et al. 1993, Racusen et al. 1999). Banff scoring for acute rejection has been shown to have clinical relevance when predicting rejection reversal and may be useful for choosing first-line therapy of rejection episodes (Gaber et al. 1996), also the Banff scoring for chronic rejection changes has been shown to correlate well with subsequent graft function and survival (Nickerson et al. 1998).

Although kidney grafts are relatively easy to biopsy, the incidence of complications related to biopsies, especially the risk of clinically significant bleeding, limits the use of routine core biopsies. Thus, less invasive means of monitoring renal allograft status have been developed. Cytologic evaluation of fine-needle aspiration (von Willebrand 1980, von Willebrand and Lautenschlager 2003), a minimally invasive procedure that can be repeated at frequent intervals, is used to evaluate the presence or absence of acute rejection and is today used especially in pediatric kidney transplantation (Their et al. 2001). Recently also methods based on competitive polymerase chain

reaction (PCR) amplification of messenger RNA has been developed to quantitate a small number of proinflammatory cytokines and cytotoxic T cell products in urine and correlate their levels with renal allograft dysfunction (Li et al. 2001).

1.4. Immunosuppressive medication

After the kidney transplantation life-long immunosuppression is necessary to prevent kidney allograft rejection. Immunosuppressive medication should be optimized to be in balance between allograft rejection and opportunistic infections as well as post-transplant malignancies. The molecular targets for the main immunosuppressive drugs are shown in Figure 1.

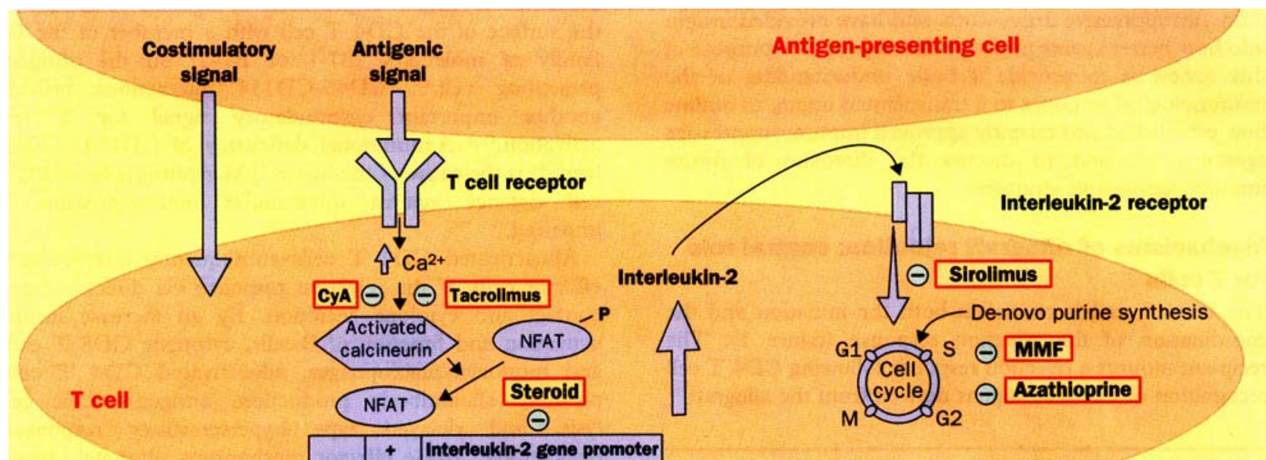

FIGURE 1. The molecular targets for the main immunosuppressive agents (Denton et al. 1999). Nuclear factor of activated T cells (NFAT). Cyclosporine (CyA). The figure is reproduced by the permission of the authors and the publisher.

1.4.1. Calcineurin inhibitors

Calcineurin inhibitors are currently the keystones of most immunosuppressive regimens used in clinical organ transplantation.

Cyclosporine is used succesfully to prevent and treat acute rejection since late 1970's (Calne et al. 1978). CsA was originally isolated form fungus imperfectus Tricoderma polysporum by Thiele and Kis in 1970. The immunosuppressive properties were discovered by Borel in 1972. CsA is a cyclic endecapeptide. It binds intracellularly to cyclophilin and the resulting complex inactivates calcineurin, a pivotal enzyme in T cell receptor signalling. Calcineurin is a serine threonine phospatase that plays a critical role in IL-2 promoter induction. Calcineurin inhibition prevents IL-2 gene transcription, thereby inhibiting T cell IL-2 production. CsA is highly lymphocyte- and particularly T- cell spesific. The therapeutic window for CsA is narrow owing to its lipophilic nature and wide inter- and intrapatient bioavailability. The main side-effects of CsA are nephrotoxicity, neurotoxicity, hepatotoxicity and gingival hyperplasia. CsA also influences glucose and lipid metabolism and causes hypertension.

Tacrolimus (FK506) is a macrocyclic lactone antibiotic that was discovered in soil samples in 1984 (Kino et al 1987). Tac is a calcineurin inhibitor like CsA. The mechanism of action of Tac is similar to CsA in that it binds to cytosolic protein, FK506 binding protein (FKBP). The Tac-FKBP complex then binds to and inhibits the activity of calcineurin. Tac was first used clinically in liver transplant patients who were suffering ongoing rejection despite CsA-based immunosuppression (Starzl et al. 1989). Currently Tac is used successfully as a maintenance immunosuppression for acute rejection prophylaxis and for rescue therapy in solid organ transplantation. Nephrotoxicity effects similar to CsA have been documented using this drug (de Mattos et al. 2000). Tac administration has been shown to be associated with a higher incidence of diabetes mellitus, whereas the incidence of hypercholesterolemia, hypertriglyseridemia and cosmetic effects (hirsutism, acne, genital hyperplasia) have been more pronounced with CsA (Pirsch et al. 1997).

1.4.2. Corticosteroids

Steroids have been used as immunosuppressive drugs since the early days of clinical transplantation (Kuss et al. 1962). Corticosteroids are non-specific anti-inflammatory drugs. The mechanism of action of steroids is complex. They inhibit cytokine production by T cells and macrophages, thereby disrupting T cell activation and macrophage-mediated tissue injury. Steroids have multiple side-effects, which were more pronounced earlier because much higher doses were needed before the combination of steroids to calcineurin inhibitors. Poor wound healing, osteoporosis, avascular necrosis, cataracts, iatrogenic diabetes, obesity and hypertension are the major side-effects of steroids. Cushingoid apperance and growth retardation limit the use of streroids, especially in children. Steroid-sparing immunosuppressive regimens are thus favourable.

1.4.3. Antiproliferative drugs

Azathioprine (AZA) has been used since the beginning of the modern era of kidney transplantation (Murray et al. 1963). AZA is an imidazole derivative of 6-mercaptopurine (6-MP). After administration it is converted to 6-MP, and further to 6-thio-inosine monophosphate. AZA-derivatives act by alkylating DNA-precursos and by inhibiting various enzyme systems. AZA is a relatively non-spesific inhibitor of cell proliferation, with side-effects from all rapidly dividing tissues, particularly from bone marrow and liver.

Mycophenolate mofetil (MMF) was originally isolated from genus penicillium. Its immunosuppressive properties were first described in 1989 (Morris et al. 1989). MMF is rapidly converted to its active metabolite mycophenolic acid, which inhibits inosine monophosphate

dehydrogenase activity and thus disables the de novo pathway for purine synthesis. MMF suppresses a wide variety of T- and B-lymphocyte responses in vitro and and in vivo.

Sirolimus (rapamycin, RPM) was first isolated from Easter Island (Rapa Nui) –derived soil microorganism Streptomyces hygroscopius. Martel and Sehgal discovered its immunosuppressive properties in 1977. Sirolimus is structually similar to Tac. Sirolimus binds to FKBP, similar to Tac, but fails to inhibit calcineurin phosphatase activity, thus it does not inhibit IL-2 production or up-regulation of IL-2 receptor. Instead, it acts downstream of calcineurin antagonists, blocking signalling events subsequent to the interaction of IL-2 with its receptor, thereby inhibiting clonal expansion of activated T cells. Safety profile of sirolimus is different from that of calcineurin inhibitors. Sirolimus is not nephrotoxic, major side-effects are hyperlipideamia and thrombocytopenia (Murghia et al. 1996).

1.4.4. Antibodies

Powerful polyclonal agents have been available since mid-1960s (Waksman et al. 1961, Woodruff and Anderson 1963). Polyclonal antibodies are produced by immunizing rabbits or horses with T cells, thymocytes or with T cell lines and used clinically to prevent or treat acute rejection. The exact mechanism of action of these polyclonal anti- T cell antibodies is not known. They are thought to kill circulating T cells rapidly after administration. All of these antibodies are highly immunosuppressive, though their activity is lost when anti-antibodies are produced. In clinical transplantation these antibodies are used for induction therapy or for the treatment of steroid-resistant rejection.

OKT3, a murine monoclonal antibody reactive with a component of the antigen-recognition complex (CD3) on T cells, was the first monoclonal antibody introduced for clinical use for rejection and induction therapy (Cosimi 1981). For long time it was considered the standard for treament of streroid-resistant rejection episodes. Other monoclonals were also introduced after OKT3, but none of them has gained wide clinical use. The problem in using monoclonals in clinics is that anti-antibodies are always produced neutralizing their effect in the long run. A toxic cytokine release syndrome has been also associated with OKT3 administration. Basiliximab and daclizumab are novel monoclonal antibodies directed against the α-chain of the IL-2 receptor which are now also in clinical use to prevent acute rejection in kidney transplantation (Adu et al. 2003). Basiliximab is a high-affinity chimeric monoclonal antibody whereas daclizumab is a humanized monoclonal antibody. Less incremental toxicity is reported with these antibodies.

1.4.5. Future regimen

There are several new immunosuppressive agents being examined in clinical trials, and as our knowledge of molecular events in immune activation improves, new targets for manipulation are discovered. Everolimus and leflunomide analog FK778 are now in clinical trials to test their usefulness in clinical transplantation. FTY720 has a unique immunosuppressive mechanism by altering lymphocyte homig, resulting in sequestration of T and B cells in lymph nodes and Peyer patches (Chiba et al. 1998). Efalizumab is a humanized monoclonal antibody preventing LFA-1/ICAM interaction, and thereby blocking T cell adhesion and activation. Campath 1H, a monoclonal antibody with potent prolonged lymphocyte-depleting properties, is now in clinical trials. There has also been a great anticipation that agents that inhibit T cell co-stimulation mediated through CD28/B7 pathways can be used to induce clinical transplant tolerance.

1.5. Outcome

Surgical complications after kidney transplantation can be divided into nonmechanical, vascular and urologic categories.

Advances in immunosupppression have decreased acute rejection episodes to the point where they are now exception rather than the rule (Cecka 1995). After CsA introduction in the late 1970's and early 1980's the incidence of acute rejection decreased dramatically, one-year survival rates for renal allografts improved from approximately 60% to between 80 to 90% (Pascual et al. 1998, Hariharan et al. 2000). However, the incidence of acute rejection in the first six months after transplantation has remained high in most centers; approximately half the recipients had at least one episode of acute rejection (Denton et al. 1999). In Finland, however, the risk for acute rejection has been less than 20%. Improvements in patient management, such as efficient bacterial and viral prohylactic agents, as well as technical advances and the introduction of monoclonal antibodies, have also been major contributors to treatment of acute rejection and the resulting improvements in 1-year graft survival (Kreis and Ponticelli, 2001).

The introduction of new immunosuppressants in the 1990's led to a decrease in the incidence of acute rejection and has improved the 1-year graft survival even more. The results of the first multicenter studies have shown that Tac has diminished the incidence of acute rejection compared to CsA (Pirsch et al. 1997, Mayer et al. 1997). An approximately 30% reduction in the incidence of acute rejection at 6 months with patients treated with tacrolimus was seen in these first multicenter studies using older formulation of CsA. The incidence of severe acute rejection with poor histological findings was also diminished when using Tac instead of CsA. Recently European

multicenter study has shown that Tac therapy was associated with a 47% relative reduction in the frequnecy of biopsy-proven acute rejection compared to CsA microemulsion during the 6-month period (Margreiter 2002). In that study also corticosteroid-resistant acute rejection confirmed by biopsy was reported in a significantly lower proportion of patients in the Tac group than in the CsA group. MMF and sirolimus have also decreased the incidence of acute rejection when combined to calcineurin inhibitors. Three large pivotal trials conducted in the United States, Europe, and a tricontinental (Europe, Canada, Australia) evaluating MMF as a part of multiple-drug regimen demonstrated approximately 30% reduction in the incidence of rejection at 6 months (Sollinger 1995, European Mycophenolate Mofetil Cooperative Study Group 1995, The Tricontinental mycophenolate Mofetil Renal Transplantation Study Group, 1996) Also sirolimus when combined to CsA reduced the incidence of acute rejection by more than 30% compared to CsA alone at both 6-month and 1-year follow-ups in Phase III trials of this new drug (MacDonald 2001).

By contrast, the long-term results are not as good as the short-term results in kidney transplantation. Chronic rejection or chronic allograft nephropathy, term preferred nowadays, is still the principal cause of late allograft loss after the first year of renal transplantation. Despite of modern immunosuppressive medication the grafts are lost due to chronic changes in an annual rate of loss of 3 to 5% (Hariharan et al. 2000). Currently there is no effective treatment available for preventing it. Thus, the development of strategies that may improve long-term outcomes by preventing late allograft loss has become a priority in renal transplantation. In clinical trials some improvement has been seen in the incidence of chronic rejection some years after transplantation in Tac-treated patients compared to CsA (Mayer et al. 1999, Vincenti et al. 2002). MMF, sirolimus and FK778 have been experimetally shown to be potential drugs to inhibit the development of chronic rejection (Räisänen-Sokolowski et al. 1995, Gregory et al. 1993, Savikko et al. 2003). However, the long-term data of these new immunosuppressants are not yet available.

2. Immunology of kidney allograft rejection

2.1. Transplantation antigens

Major histocompatibility complex (MHC) is the key determinant of immunological reactivity in transplantation (Bach and Rood, 1976). MHC consists of class I and class II molecules. These antigens are encoded predominantly by highly polymorphic loci within the major histocompatibility complex on the short arm of chromosome 6 (Gaston et al. 1995). The HLA molecules of the class I and class II are heterodimers, i.e. they consist of two different polypeptide chains, the α – and the β-

chains, which are noncovalently bound. The main function of MHC molecules is to present antigen to the T-cell receptors of CD4 and CD8 T cells. CD4 engages class II and CD8 class I by binding to a spesific loop on the side of the MHC molecule.

Class I molecules are expressed on the surface of almost all cells with a nucleus, while class II molecules have a restricted expression (Daar et al. 1984a, b). The latter are mainly found on cells which have immunological functions such as macrophages, B lymphocytes, dendritic cells, Langerhans' cells and Kupffer cell in the liver. Nonimmune cells, such as endothelial cells, express few to no class II molecules constitutively, but can be induced to do so by exposure to interferon- γ (IFN-γ) and potentially other cytokines, such as tumor necrosis factor (TNF) (Glimcher and Kara 1992).

Minor histocompatibility antigens may play a prominent role in graft rejection in a recipient who is given a MHC compatible graft but where pre-existing sensitization to minor histocompatibility antigens exists (Dallman and Morris 1988). This can be demonstrated in rats and mouse (Fabre and Morris 1975, Peugh et al. 1986) and probably explains the frequent rejections seen in renal transplants between HLA identical siblings. The structure and distribution of minor histocompatibility antigens has been difficult to assess. This is due mainly to inability to raise antisera directed at minor histocompatibility antigens which makes analysis of the expression and function of these molecules extremely difficult.

2.2. T-cells

The spesific immune response in the graft is mediated by T-cells (Fig. 2.). The CD4 T cell has generally been found to be critical in initiating rejection (Mason et al. 1984, Krieger et al. 1996), while the role CD8 T-cells is not fully elucidated (Bueno and Pestana, 2002). The CD4 T cell is crucial in both the initiation and the coordination of the rejection response.

T cells are activated after recognition of foreign antigens derived from the allograft. This can happen either directly or indirectly. Recipient T cells may recognise intact foreign MHC encoded molecules on donor cells (direct allorecognition) or peptides derived from foreign MHC molecules, shed from the graft and subsequently processed and presented, bound to self MHC molecules by recipient antigen presenting cells (indirect allorecognition) (Sayegh et al. 1994). In the direct pathway T cell receptor (TCR) directly recognizes an intact allo-MHC molecule expressed on donor cells. Direct allorecognition can activate a much larger proprtion of the T cell pool than indirect allorecoginition and may cause the vigorous immune response in acute rejection (Liu et al. 1993). In the indirect

pathway, donor MHC alloantigens, either class I or clas II molecules, are first engulfed by recipient antigen-presenting cells (APCs) before being processed and presented as MHC-derived peptides to CD4 T cells in the context of recipient MHC class II molecules. APC is any cell that expresses peptide-MHC complexes that can be recognized by specific T cells (Austyn and Wood 1993). For instance macrophages, dendritic cells and endothelial cells can act as APCs. Indirect allorecognition generates smaller numbers of alloreactive T cell clones, and several lines of evidence suggest that this pathway may lead to insidious immune response that occurs in chronic rejection (Vella et al. 1997).

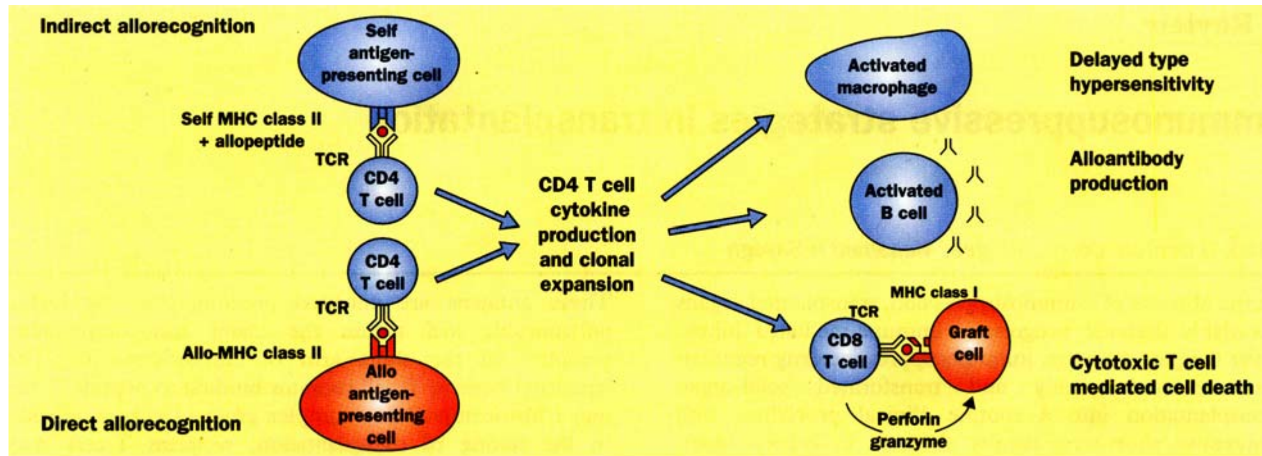

FIGURE 2. Cellular interactions in anti-allograft response (Denton et al. 1999). The figure is reproduced by the permission of the authors and the publisher.

The primary requirement for T cell activation is ligation of its antigen-spesific receptor, TCR, by antigen peptide contained with MHC-protein. The TCR comprises two similar chains, the α and β chains, which are complexed to several more chains of the CD3 complex. The TCR confers specificity of antigen/MHC binding whilst the CD3 complex transduces a signal of activation to the T cell.

Antigen recognition alone is insufficient to activate fully the CD4 T cell. Other requirements for T-cell activation are costimulation by other stimulatory molecules and the presence of cytokines. A second (costimulatory) signal must be provided by cognate ligands on the antigen-presenting cell. Without such signals, not only is activation incomplete, but also T cells become unresponsive to further antigenic stimulation, a state that is called anergy (Schwartz, 1990). Since T cell is central to immune reponses, anergy would in fact be a desirable state in transplantation. The best-characterized costimulatory signal is provided by ligation of CD28 on the surface of the CD4 T cell within a member of the B7 family of molecules, B7-1 or B7-2 on the antigen-presenting cell (Linsley et al. 1993, Sayegh et al. 1998). CD40-CD154 interactions provide another important costimulatory signal for T cell activation (Durie et al. 1994, Denton et al. 1998). Of importance is

that signals delivered through CD28/CTLA-4 are insensitive to the inhibitory effects of CsA and therefore result in CsA-resistant cytokine expression. In addition to this large number of cell-cell based interactions, T cells also receive important signals through the binding of cytokines to specific cell surface cytokine receptors.

When all signals are provided, T cell secretes optimum concentrations of interleukin-2 (IL-2) – a potent autocrine growth factor that induces T cell proliferation, clonal expansion and cytokine production. Via secretion of various cytokines and direct cell-cell contact alloactivation of CD4 T cells subsequently leads to terminal differentiation and proliferation of B-cells, cytotoxic CD8 T-cells, natural killer (NK)- cells and macrophages. The influence of elements such as route and dose of antigen delivery and the presence of co-factors drives the cytokine response of T CD4+ cells towards either Th1 or Th2 pattern. Th1 cells, through the cytokines they produce, tend to drive a cell-mediated response, although IL-2 and IFN-γ may also be required for B-cell proliferation and differentiation, whereas Th2 cells direct the immune system to an antibody-dominated response. By an increase in the activation and function of B cells, CD8 T cells and monocyte/macrophages, alloactivated CD4 T cells promote alloantibody production, antigen-spesific cell lysis, and delayed type hypersensitivity responses, respectively. These effector functions result ultimately in tissue damage in graft rejection.

The two main cytotoxic mechanisms in graft destruction are the perforin/granzyme and the Fas/Fas-ligand (FasL) system (LeMoine et al. 2002). The perforin/granzyme pathway is used first and foremost by CD8+ T cells and NK cells. Activated CD8 T cells synthesize perforin and granzymes, which are then targeted to intracellular cytotoxic granules (Shresta et al. 1998). Perforin molecules insert within the allogenic cell membrane and form polymers that create channels, through which granzymes A and B penetrate into the cytoplasm. There, granzymes can either directly enter within nucleus or they may cleave cytoplasmic procaspases into caspases, which will then also move into the nucleus (Graubert and Ley 1996). Caspases are a family of cysteine proteases that cleave aspartate residues from many substrates including the caspases themselves. Caspase activation is responsible for the appearance of functional nuclease activity that finally triggers DNA fragmentation and leads to apoptosis (Graubert and Ley 1996, Heibein et al. 1999). Fas/FasL interaction is the most important mechanism for CD4 CTL-mediated cytotoxicity (Kagi et al. 1996). Wheras Fas, a member of the TNF family of death receptors, is constitutively expressed on most cell surfaces (Peter and Krammer 1998), FasL is essentially inducible. It seems that FasL expression is restricted to Th1 and not Th2-type alloreactive T-cells (Kagi et al. 1996, Matesic et al. 1998). On the cell surface, FasL is rapidly cleft by a metalloproteinase and shaded into a trimmer that binds

Fas on the target cell surface. Fas engagement results in the death-inducing signal complex formation and the activation of caspase cascade, which will ultimatley induce target cell apoptosis similar to that induced by the perforin/granzyme system.

2.3. B-cells, alloantibodies

B-cells are activated by direct interaction of native antigens with their surface antigen receptors. As with T-cell activation co-stimulatory signals are needed for B-cell activation. These signals may be in the form of cell-cell interaction and cytokines. Signals delivered through the CD19/CD21 complex and CD40 seem to be most critical of the cell-cell interactions. Of cytokines IL-2, IL-4, IL-5, IL-6 and IL-10 are probably the most important for B-cell proliferation and differentiation. Activation of B-cells leads, in the presence of co-stimulatory signals, to proliferation of B-cells and to their differentiation into plasma cells that secrete large amounts of antibody. Other B cells differentiate into memory B cells (Campbell and Halloran 1996).

Many of the changes associated with acute rejection, such as arteriolar thrombosis, interstitial hemorrhage and fibrinoid necrosis of the arteriolar walls, may be mediated through the deposition of antibody and fixation of complement. Antibody may also cause tissue damage through activitity of killer cells in antibody dependent cellular cytotoxicity. Many different leukocytes appear to be able to express killer activity. The antibody acts as a bridge between the targer tissue and the effector cell, activating the lytic machinery of the killer cell and thus resulting in tissue damage (Perlmann et al. 1969). In vitro studies have shown that anti-HLA antibodies may affect the expression of growth factor receptors on vascular wall cells and, through such a mechanism, graft atherosclerosis (Bian et al. 1998, Harris et al. 1997).

2.4. Monocyte –macrophages

Monocyte-macrophages play also a central in allograft rejection. They do not express antigen-specific receptors. Their activation requires cytokines, which are provided by alloreactive T lymphocytes. IFN-γ is the major stimulus for macrophage activation, TNF and IL-4 are also capable to activate macrophages (Mosser 2003). After activation macrophages produce toxic molecules such as nitric oxide (NO), oxygen intermediates and TNF-α (Le Moine et al, 2002). NO, a highly reactive nitrogen metabolite, is cytotoxic at high concentrations. TNF-α induces target cell apoptosis or necrosis through caspase activation. Macrophages are also capable to produce and secrete various enzymes, inflammatory mediators and growth factors. Mononuclear cell infiltrates are especially typical for acute vascular rejection (von Willebrand et al, 1992). Macrophages in the

graft are also shown to be a predisposing factor for to the development of chronic changes (von Willebrand et al. 1992, Croker et al 1996).

2.5. Natural killer-cells

NK-cells belong to the innate arm of the immune response because they have spontaneous cytotoxic activity against a variety of target cells in an MHC-unrestricted way. Thus, cells with NK activity can kill targets that do not express classical MHC molecules. Moreover, NK cells do not show secondary or memory responses. Cytoxic acitivity of NK-cells can be triggered within a few minutes of encountering an appropriate target. However, NK cells can be activated by cytokines to a different state in which they have greater cytotoxic activity (Austyn and Wood 1993). While clearly a potent source of cytotoxic activity, a role for the NK cell in allograft rejection remains to be firmly established. Although NK cells are unlikely to have a major direct role in solid organ allograft rejection, they may stimulate T- or B-cell activity (Snapper et al. 1993). On the other hand, NK cells have quite clearly been shown to be involved in the rejection of bone marrow transplants (Yu et al. 1992).

2.6. Cytokines and adhesion molecules

Cytokines are a key factor in modulating the immunological responses in rejection processes, they are involved both in the antigen presentation as well as in the effector phases of the immune response against an allograft. They are relatively low molecular mass proteins that act on receptors on the target cells. Cytokines can be grouped into families based on their structure and that of their receptors. The principal cytokines are classified as haematopoietins, interferons (IFN), chemokines, TNFs and transforming growth factors (TGF).

Various cytokines direct Th1 or Th2 expansion during the initial steps of CD 4+ T cell activation, which therafter results in different cytokine secretion profiles in these cells. IL-12 and IFN-γ promote Th1 differentiation. Th 1 cells produce IFN-γ and IL-2; in graft rejection, this will result in the activation of CD 8 cytotoxicity, macrophage-dependent delayed type hypersensitivity, where macrophages release toxic molecules, and the synthesis of complement-fixing immunoglobulin G2a antibody by B cells (LeMoine et al. 2002). Th2 cells secrete IL-4, IL-5, IL-9, IL-10 and IL-13. This will mainly trigger eosinophil activation. Activated eosinophils release granules that contain several harmful enzymes such as major basic protein, eosinophil-derived neurotoxin, eosinophil cationic protein and eosinophil peroxidase that are responsible for tissue destruction (Assa'ad et al. 2000). Chemokines are a superfamily of small proteins that direct leukocyte migration and position (Rossi and Zlotnik 2000). The release of chemokines, for example RANTES and macrophage-

inflammatory protein-1, by the transplant itself guide alloreactive T cells and circulating blood leukocytes into the allograft.

IFN-γ and TNF-α mediate macrophage and endothelial cell activation as well as MHC and adhesion molecule induction in endothelial and epithelial cells. TNF-α also mediates T cell cytotoxicity and causes capillaries to leak. TGF-β induces fibroblast growth and collagen formation, it also has immunosuppressive effects (Letterio and Roberts 1998). TGF-β is a key fibrogenic cytokine involved in the fibrosis of a number chronic diseases of the kidney and other organs (Border and Noble 1995) and recently evidence has shown that TGF-β is involved in the pathogenesis of chronic allograft nephropathy (Sharma et al. 1996, Shihab et al. 1996). In addition TGF-β modulates the fibrogenic actions of bFGF and PDGF (Klahr and Morissey 2000). Production of TGF-β in the development of chronic allograft nephropathy may be modulated by intrarenal renin-angiotensin system as Angiotensin II induces TGF-β1 production and secretion by the mesangial cells (Border and Noble 1998). In addition TGF-β is upregulated during the development chronic allograft nephropathy by direct effect of CsA, which stimulates the synthesis and expression of TGF-β1 (Khanna et al. 1997).

Adhesion molecules are crucial for leukocyte migration into the graft tissue. They have also an important role both in initiation and maintaining the inflammatory response of rejection. Adhesion molecules also play a role in T cell activation (Altmann et al. 1989). Three major structural groups of adhesion molecules are traditionally recognized as selectins, integrins and immunoglobulin superfamily (Table 1.). There are also adhesion molecules not belonging to these groups such as VAP-1 and CD44 (Salmi and Jalkanen 1997).

TABLE 1. Adhesion molecules in lymphocyte adhesion to endothelium

Adhesion molecule	Family	Ligand	Family
P-selectin (CD62P)	selectin	PSGL-1 (CD162)	sialomucin
E-selectin (CD62E)	selectin	CLA, ESL-1, PSGL-1	
L-selectin (CD62 L)	selectin	MAdCAM-1	immunoglobulin
		PNAd, GlyCAM-1	sialomucin
		CD34, sulfated sLex	
ICAM-1(CD54)	immunoglobulin	LFA-1(CD11a/CD18)	integrin
ICAM-2 (CD102)	immunoglobulin	LFA-1(CD11a/CD18)	integrin
ICAM-3 (CD0)	immunoglobulin	LFA-1(CD11a/CD18)	integrin
VCAM-1(CD106)	immunoglobulin	VLA-4 (CD49e/CD29)	integrin
PECAM-1(CD31)	immunoglobulin	CD31	immunoglobulin
VAP-1		unknown	
hyaluronate		CD44	proteoglycan

Leukocyte adhesion to activated endothelium proceeds in a cascade-like manner: initial weak contacts leading to tethering and rolling of leukocytes on endothelium are between selectins and their ligands. This allows leukocyte to sample to the endothelial cell surface microenvironment where high concentrations of chemokines are sequestered. Subsequent firm adhesion between leukocytes and endothelium is mediated by activated integrins on the surface of leukocytes. This is followed by transmigration of leukocytes through endothelium towards the site of inflammation (Springer 1995). Interactions of VLA-4/VCAM-1 and LFA-1/ICAM-1 are of indisputable importance in acute rejection of kidney tranplants (Solez et al. 1997). Active L-selectin ligands are induced during acute renal allograft rejection as well (Kirveskari et al. 2000). Up-regulation of peritubular capillary VCAM-1 has also shown to be diagnostic for chronic rejection (Hill et al. 1995, von Willebrand et al. 1997).

3. Chronic allograft nephropathy

3.1. Clinical manifestations and diagnosis

In the past, chronic renal allograft rejection has been widely viewed predominantly as immunologically based. Increasing evidence, however, indicates that chronic rejection is a multifactorial process in which immunologic and nonimmunologic factors contribute to the progressive demise of renal graft function. Because it has become apparent that multiple factors play a part in chronic rejection, the more inclusive term “chronic allograft nephropathy” has been introduced (Halloran et al 1999). Chronic allograft nephropathy is manifested clinically by gradual decrease in renal function accompanied by hypertension and low-grade proteinuria, usually occurring months or years after transplantation (Morris 1999, Halloran et al. 1999, Monaco et al. 1999).

Chronic allograft nephropathy is diagnosed histologically in biopsies. Interstitial inflammation and fibrosis, vascular changes, glomerular mesangial matrix increase and tubular atrophy are characteristic histopathological features in chronic allograft nephropathy (Isoniemi et al. 1992) (Fig. 3). Banff classification is developed from an international consensus discussion to standardize renal allograft biopsy interpretation (Solez et al. 1993, Racusen et al. 1999). Chronic allograft damage index (CADI) is developed to show the intensity of chronic changes in the transplant as a single numerical figure (Isoniemi et al. 1992). Several studies suggest that serum creatinine values may serve as surrogate markers for the identification of renal transplant recipients at increased risk for

the development of chronic rejection (Monaco et al. 1999) although the sensitivity of this assay is not high. Low-grade proteinuria might also be seen in chronic rejection (Russell 1997).

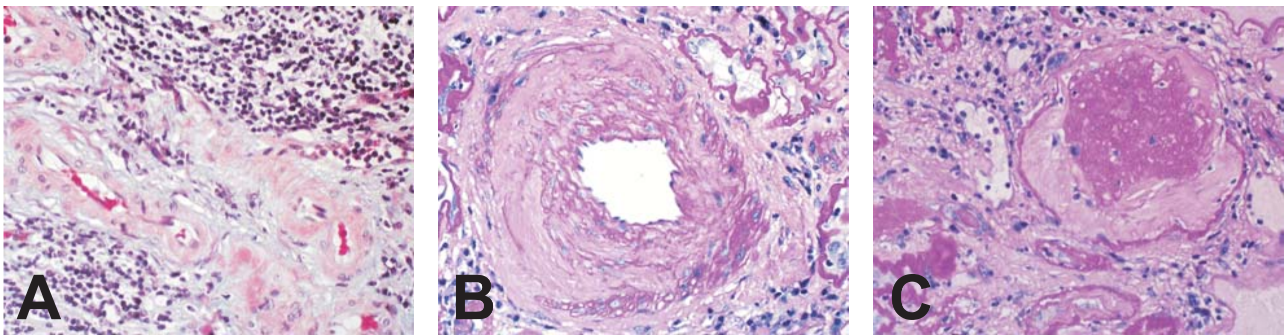

FIGURE 3. Histological changes typical for chronic allograft nephropathy. A) Interstitial inflammation and fibrosis, B) arterial intimal proliferation and tubular atrophy, C) glomerular mesangial matrix increase and glomerular sclerosis.

3.2. Risk factors

Causes of chronic allograft nephropathy can be classified as alloantigen-dependent and alloantigen-independent.

Alloantigen-dependent factors involved in the process of chronic rejection include HLA mismatching, ongoing alloresponsiveness modulated by immunosuppression and acute rejection (Kreis and Ponticelli 2001).

Acute rejection is the single most important risk factor for the development of subsequent chronic rejection (Yilmaz et al. 1993, Troppman et al. 1995). Acute rejection episodes occurring more than 2 months posttransplantation resulted in marked increases in the incidence of biopsy-proven chronic rejection (Basadonna et al. 1993). Severity of acute rejection episodes also impacts long-term graft survival and affects the propensity to develop chronic rejection (Chavers et al. 1995, Humar et al. 1999, Dickenmann et al. 2002). An exact histological differential diagnosis also permits conclusions to be drawn. Interstitial infiltration yields a much better prognosis than vascular rejection, which reduces 9-year graft survival by >50% (van Saase et al. 1995). Patients experiencing even a single acute rejection episode should be considered at risk for chronic rejection (Matas et al. 1994). Moreover, patients experiencing late or multiple rejection episodes are at even greater risk for the development of eventual chronic rejection (Humar et al. 1999). Thus, every effort should be made to prevent the first acute rejection episode and to minimize the likelihood of subsequent episodes to improve long-term clinical outcome (Monaco et al. 1999).

The incidence of chronic rejection increases with increased HLA mismatching. If a patient is receiving inadequate immunosuppression, chronic rejection may ensue from production of a permanent alloimmune response to the mismatched kidney. Association between anti-HLA antibodies and chronic rejection has been demonstrated suggesting that antibodies produced against mismatched HLA antigens are part of the chronic rejection pathogenesis (Suciu-Foca et al. 1991). Inadequate immunosuppression has been shown to have substantial impact on long-term renal allograft function (Salomon, 1992). Apart from clinical acute rejection, patients may have subclinical rejection that causes ongoing immunologic injury leading to chronic rejection (Rush et al. 1995, Rush et al. 1998). On the other hand, nephropathy associated with long-term use of calcineurin inhibitors generates similar changes such as tubular atrophy, interstitial fibrosis and vasculopathy. These histological changes are difficult to distinguish from histological changes induced by chronic rejection, and herein lies the dilemma when using calcineurin inhibitors for immunosuppression.

Alloantigen-independent factors have become more prominent as alloantigen-dependent causes of chronic allograft nephropathy have diminshed, most likely due to improved immunosuppression. Common sources of alloantigen-independent-related chronic allograft nephropathy are poor graft quality pretransplantation, delayed graft function, ischemia/reperfusion injury, infections, use of calcineurin inhibitors and possibly cardiovascular risk factors (Kreis and Ponticelli 2001).

Donor kidneys experience a series of potentially damaging ischemic events during organ retrieval, storage and transplantation. Following ischemic injury, release of humoral mediators and leukocyte-endothelial cell interactions promote leukocyte infiltration into the allograft. Experimental studies have shown that ischemia-reperfusion related injury causes up-regulation of various adhesion molecules, cytokines and growth factors as well as increases in oxygen radicals (Azuma et al. 1997, Daemen et al. 1999, Waltenberger et al. 1996, Wanders et al. 1995). Long ischemia time before revascularization of kidney transplant may also cause acute tubular necrosis.

Donor age has also shown to be a risk factor for the development of chronic allograft nephropathy. The limited availability of organs has prompted many centers to use organs from older cadaveric donors. Kidneys from such donors have a lower survival rate than those from younger cadaveric donors (Alexander et al. 1994, Hariharan et al. 1997).

Among posttransplant patients viral infections are the most important class of infection, with cytomegalovirus representing the most critical type. CMV has been associated with both acute, life-

threatening disease and long-term complications, such as chronic rejection (Rubin 2001). CMV infections are related to rejection, patients with frequent rejections having more CMV infections, while patients with CMV infection have more rejections (von Willebrand and Lautenschlager 2003). CMV infection has been shown to induce HLA class II antigens in the kidney transplant (von Willebrand et al. 1986) Apparently this is the link to the rejection process. Other viral infections may also negatively impact transplantation outcome, including Epstein-Barr, hepatitis B and C, human herpesvirus 6 and community-acquired viruses (Söderberg-Nauclér and Emery, 2001).

Posttransplant hypertension is a common feature in renal transplant recipients and has been associated with chronic allograft nephropathy in a number of studies (Opelz et al. 1998, Peschke et al. 1999, Sorof et al. 1999). Whether posttransplant hypertension is a cause or an effect remains unknown (Sanders et al. 1995). As calcineurin inhibitors and corticosteroids are risk factors for posttransplant hypertension this is even more complicated (Mihatsch et al. 1998, Veenstra et al.1999). Calcineurin inhibitors may promote hypertension through direct vasoconstrictive effects on smooth muscle cells (SMC) (Fellström et al 1998), these effects are more pronounced by CsA compared to Tac.

Dyslipidemia is also associated with deterioration of renal allograft function (Isoniemi et al. 1994). Oxidatively modified LDL cholesterol may be the most important hyperlipidemic factor in the development of chronic allograft nephropathy (Fellström 2001).

3.3. Mechanisms

The histological changes characteristic for chronic allograft nephropathy were described already at the early days of clinical kidney transplantation (Hume et al. 1955). They were first thought to be due to an increased pressure in the vessels of transplanted kidneys, soon it came evident that these pathological changes were due to immunological processes as opposed to hemodynamic factors (Porter et al. 1963, Kincaid-Smith 1964). According to the current knowledge both immunologic and nonimmune mechanisms contribute to the development of chronic allograft nephropathy. Immunologic mechanisms seem mostly responsible for the injury and subsequent tissue response while nonimmune mechanims act mostly as progression factors (Paul, 2002).

Vasculopathy or graft vessel disease is a prominent histological feature of chronic allograft nephropathy. Renal transplant vasculopathy begins when arteries are damaged either by alloimmune-dependent or alloimmune-independent factors. Subsequently arterial vascular injury invariably leads to vascular remodeling and concentric luminal narrowing prodced by intimal

thickening. The typical scenario involves inflammation at the site of injury accompanied by the release of cytokines and growth factors that function as chemical mediators, stimulating smooth muscle cells to proliferate and migrate to the intima (Morris 2001). Recently it has been demonstrated that proliferating intimal cells in transplant arteriosclerosis originate from recipient bone marrow (Shimizu et al 2001, Sata et al. 2002). Once in the intima, these cells are driven to further proliferation, with deposition of extracellular matrix. The result is intimal thickening, flow obstruction and tissue ischemia. Proliferative processes involving stimulation by cytokines or growth factors may also characterize other histopathological features of chronic allograft nephropathy.

It is hypothesized that acute rejection could cause the primary injury leading to induction of reparative mechanisms resulting in fibrosis and mesenchymal cell proliferation. During acute rejection episode antibodies, complement, cytokines and leukocytes all participate to augment the inflammatory reaction. Injuries incurred during organ preservation and storage as well as stress responses in local tissues after an acute rejection episode can promote the release of potent chemokines and induce infiltration at the site of injury. In this sense, acute rejection is viewed not only as an immunologic risk factor but also as the most potent source of injury to the graft.

Regardless of the triggering event, however, the subsequent steps in the cascade may be more or less the same during the development of chronic allograft nephropathy. All will eventually result in graft inflammation. A large number of genes are differentially expressed during the development of chronic allograft nephropathy. These consist of immune response-related genes, adhesion molecules and their receptors, cytokines and chemokines, vascular smooth muscle cell growth factors and their receptor genes as well as genes controlling vasoactive hormones, such as endothelin-1, matrix metalloproteinases, tissue inhibitors and inducible nitric oxide synthase (Häyry et al. 1999).

Although the molecular mechanisms of chronic rejection remain largely unknown, there is some evidence that the humoral response may also be involved in its pathogenesis. Ig and complement deposits are found in areas of intimal thickening and the pathological changes can be reproduced by intra-arterial infusion of donor-spesific antisera (O´Conell and Mobray 1973). More recently direct evidence for the involvement of alloantibodies in chronic rejection has been provided by studies in which experimental animals with selected congenital or genetically manipulated immunological deficiences were used (Russell et al. 1997, Hancock et al. 1998). The exact mechanism of the antibody action is not known but it may involve complement, not only as lytic but also as an activating agent. Terminal complement C5b-9 proteins can elicit signals for cell proliferation by

releasing growth factors from cultured human endothelial cells (Benzaquen et al. 1994). C4d deposits have also been demonstrated in peritubular capillaries of human renal transplants with chronic rejection (Mauiyyedi et al. 2001). C4d is a fragment of the classical complement pathway component C4, which is activated by antigen-antibody complexes (Chakravarti et al. 1987). In addition a close association of peritubular C4d deposition and monocyte/macrophage infiltration has been demonstrated already in acute renal allograft rejection indicating a poor graft survival (Magil and Tinckam 2003).

3.4. Treatment

The use of both immunologic and nonimmunologic strategies should be implemented to minimize chronic rejection risk factors and treat associated conditions, such as hypertension. Although CsA has improved the short-term results it has failed to significantly improve the long-term results. There is even evidence that the accelerated form of transplant arteriosclerosis and fibrosis in the kidney, heart and liver may be linked to the administration of CsA, even within therapeutic levels (Sommer et al. 1985, Demetris et al. 1985, Paavonen et al. 1993). In rat aortic allograft model of chronic rejection CsA is shown to induce accelerated allograft arteriosclerosis (Mennander et al. 1991).

The effect of Tac on the development of chronic changes is yet unknown in the long run. The results of first multicenter studies have shown that some improvement is seen in the incidence of chronic rejection some years after transplantation in Tac-treated patients compared to CsA-treated patients (Mayer 1999, Vincenti 2002). Although Tac is a calcineurin inhibitor like CsA, these drugs are structurally different and thus likely to have also functional differencies. There are studies demonstraing that Tac may exert a less fibrogenic influence on renal transplants (Mohamed et al. 2000, Bicknell et al. 2000, Baboolai et al. 2002, Waller et al. 2002). A potential advantage of Tac over CsA is that it appears to be associated with a lower incidence of hypertension and hyperlipidemia (Pirsch et al. 1997).

MMF has been shown to prevent chronic rejection of rat kidney allografts, and thus suggesting it to be a very promising agent (Azuma et al. 1995). However, long-term follow-up studies of treament with mycophenolate mofetil in the United States have not revealed any effect of this drug on graft survival or the prevalence of chronic rejection (Renal Transplant Mycophenolate Mofetil Study Group 1999).

Sirolimus is structurally releated to tacrolimus but it is not calcineurin inhibitor like CsA and Tac, in addition no nephrotoxic side-effects related to calcineurin inhibitors have been described with

sirolimus. Experimentally sirolimus has been shown to inhibit neointima formation (Gregory et al. 1993), and thus it is a promising agent for preventing chronic rejection. In addition excellent results have been seen in sirolimus-coated stents in preventing restenosis after angioplasty in humans (Degertekin et al. 2003). Combined to calcineurin inhibitors acute rejection incidence has decreased with sirolimus in kidney transplantation (MacDonald 2001,Yang 2003). Also good 1-year transplant outcomes have been received by combining sirolimus to basiliximab and by avoiding calcineurin inhibitors (Flechner et al. 2002). The long-term results of these combinations are not yet available.

Leflunomide (LFM) has been shown experimentally to inhibit both acute and chronic allograft rejection and restenosis (Waer 1998). FK778 is a LFM analogue with shorter half-life (Silva and Morris 1997), and thus a more potential drug for clinical use than LFM, which has a long half-life up to 4 weeks. Previously it has been demonstrated that FK778 inhibits vascular response to injury via a mechanism, which is likely independent of its immunosuppressive effect (Savikko et al. 2003). FK778 is now in first clinical trials in kidney transplantation, and it is described as “the rising star” in preventing chronic allograft nephropathy (Williams 2002 and 2003).

Although no immunosuppressive regimen has been shown to be effective for the treatment or prevention of chronic allograft nephropathy in humans so far, there are several strategies to prevent late allograft loss. Minimizing early allograft injury by improving perioperative management, pharmacologic prevention of acute rejection, treatment of severe or refractory acute rejection, definition of the optimal long-term dose of calcineurin inhibitor, discontinuation of corticosteroids in patients with stable condition, and treatment of hypertension and hyperlipidemia are currently used for optimizing late allograft function. In experimental rat kidney transplantation models a combination of angiotensin II receptor antagonist with MMF (Noris et al. 2001) and pravastatin (Ji et al. 2002) have prevented chronic rejection. In the future also specific inhibition of certain growth factors, adhesion molecules and cytokines may be effective in preventing chronic allograft nephropathy.

3.5. Animal models

Chronic allograft nephropathy is a disease that in humans extends over months and years before becoming fully developed. In animal models of CAN, impairment of renal function and histopathology similar to CAN can be produced in weeks. However, we cannot assume that the pathogenesis of CAN in these models excactly reflets the pathogenesis of CAN in human renal transplant recipients, and thus these models have limitations. Results from animal studies may, however, provide us with a better understanding of human disease process.

Renal transplantation between Fisher 344 and Lewis rats is a well-established model for chronic renal allograft rejection (White and Hildeman 1968, Diamond et al. 1992). In this model a weakly histocompatible combination allows rats to survive spontaneously, without the use of immunosuppressive drugs. Renal transplantation between Dark Agouti and Wistar Furth rats is another histoincompatible model for chronic allograft nephropathy (Yilmaz et al. 1992, Savikko et al. 2002 and 2003). In this model rats require clinically relevant immunosuppression to survive, the situation reflecting more the situation in man.

4. Platelet-derived growth factor

4.1. Ligands and receptors

Platelet-derived growth factor (PDGF) is one of the most ubiquitous of the peptide regulatory growth factors. Originally, PDGF was identified as a constituent of whole blood serum that was absent in cell-free plasma-derived serum (Kohler and Lipton 1974, Ross et al. 1974, Westermark and Wasteson 1976); PDGF was subsequently purified from human platelets (Antoniades et al. 1979, Heldin et al. 1979, Deuel et al. 1981, Raines and Ross 1982).

PDGF is a family of cationic homo- and heterodimers of disulfide-bonded A- and B-polypeptide chains. The genes for the A- and B-chains for PDGF are located on chromosomes 7 and 22, respectively (Betsholtz et al. 1986, Dalla Favera et al. 1982, Swan et al. 1982). The mature parts of the A- and B-chains of PDGFs are ~100 amino acid residues long containing a characteristic motif of 8 cysteine residues and show ~60% amino acid sequence identity. Eight cysteine residues are perfectly conserved between the two chains (Heldin and Westermark 1999). Approximatley 70% of the PDGF purified from human platelets consists of PDGF-AB and the rest is mainly PDGF-BB (Mannaioni et al. 1997). Recently two novel PDGF ligands –C and –D were also found (Li et al. 2000, Bergsten et al. 2001, LaRochelle et al. 2001). Human PDGF-C and PDGF-D genes are located on chromosomes 4q32 and 11q22.3, respectively (Uutela et al. 2001). PDGF-C and PDGF-D are polypeptides of 345 and 370 amino acid residues, respectively, with a highly conserved pattern of eight cysteine residues (Li and Eriksson 2003). They share an overall homology of 43%.

PDGFs are structurally similar to the vascular endothelial growth factor (VEGF) family (Joukov et al, 1997). PDGF-BB has been crystallized and its three-dimensional structure solved at 3.0 Å resolution (Oefner et al. 1992). The three-dimensional structure of PDGF-BB is not only similar to that of VEGF, which has a related amino acid sequence (Muller et al. 1997), but also shows some

resemblance to those of nerve growth factor and transforming growth factor-β (TGF- β), despite the fact that the latter factors have no sequence similarity with PDGF (Murray-Rust et al. 1993). The *sis* oncogene of simian sarcoma virus (SSV) is related to the B-chain of PDGF, and SSV transformation involves autocrine stimulation by a PDGF-like molecule (Keating and Williams 1988).

Although the α-granules of platelets are a major storage site for PDGF, recent studies have shown that several different cell types express PDGF, most often both the A and the B polypeptide chains (Table 2). However, less is known about which cell types synthesize the newly discovered C and D chains. In human adult tissues, 2.8 and 3.9 kb PDGF-C mRNAs and 4.0 kb PDGF-D mRNA are expressed in many organs with high levels in heart, pancreas, kidney and ovary. PDGF-D expression is generally lower, and more restricted when compared to PDGF-C expression (Li and Eriksson 2003).

TABLE 2. Normal cell types expressing PDGF

Cell type	PDGF-A	PDGF-B	Reference
Platelets/megakaryocytes	x	x	Kaplan et al. 1979
Macrophages	x	x	Shimokado et al. 1985
Fibroblasts	x	x	Paulsson et al. 1987
Vascular smooth muscle cells	x	x	Nilsson et al. 1985
Vascular endothelial cells	x	x	Dicorleto and Bowen-Pope 1983
Kidney mesangial cells	x	x	Abboud et al.1987
Keratinocyts	x	x	Heldin and Westermark 1999
Skeletal myoblasts	x		– " –
Neurons	x	x	– " –
Astrocytes	x		– " –
Schwann cells	x	x	– " –
Retinal pigment epithelial cells	x	x	– " –
Leydig cells	x	x	– " –
Oocyte, blastocyst	x		– " –
Placental cytotrophoblasts	x	x	– " –
Uterus endometrium/myometrium	x	x	– " –
Mammary epithelial cells	x	x	– " –

Both the A-chain and the B-chain of PDGF family are synthesized as precursor molecules, which need to undergo proteolytic processing before they can act on their target cells (Östman et al. 1992), also C- and D-chains are prorease-activated (Li et al. 2000, Bergsten et al. 2001, LaRochelle et al. 2001). The A and B chains are independently regulated, and the synthesis is often induced in response to external stimuli such as trombin in endothelial cells (Daniel et al. 1986, Harlan et al. 1986), mechanical strain in vascular smooth muscle cells (Ma et al. 1999), low oxygen in several different cell types (Kourembanas et al. 1997) and by various growth factors and cytokines.

PDGF receptor occupation by ligand leads to receptor dimerization allowing for autophosphyrylation and subsequent association of receptor kinase substrates (Fig. 4.). The A and C chains of PDGF bind α receptors, whereas the B chain binds both α and β receptors with high affinity (Heldin and Westermark 1999, Li et al. 2000). Thus, PDGF AA and PDGF CC induce αα receptor dimers, PDGF AB αα or αβ receptor dimers, and PDGF BB all three possible types of dimers. PDGF-DD is a spesific agonist ligand for PDGFR-β (Bergsten et al. 2001).

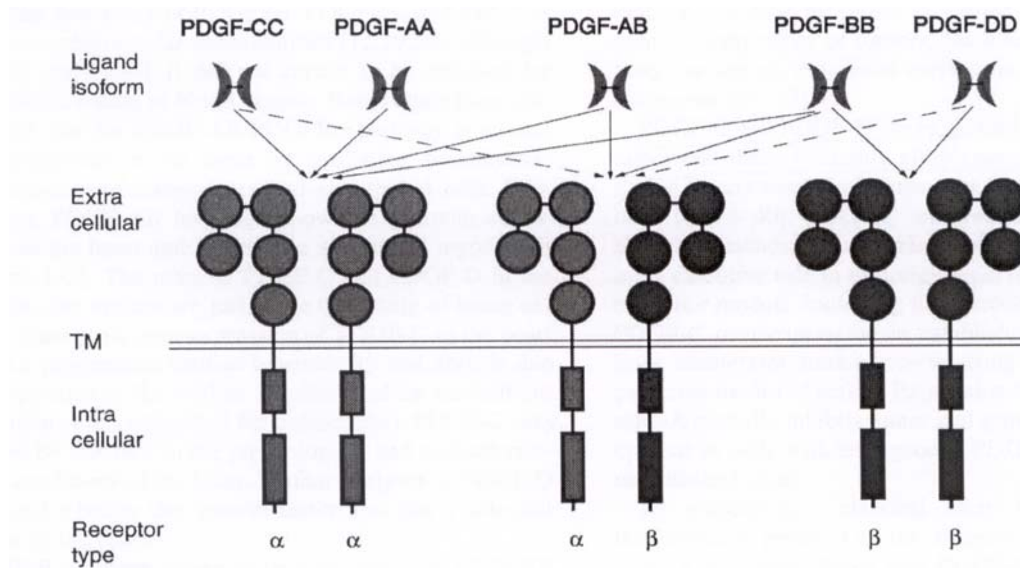

FIGURE 4. Receptor binding specificity of the five PDGF isoforms (Li and Eriksson 2003). The figure is reproduced by the permission of the authors and the publisher.

PDGF α and β receptors are structurally related protein tyrosine kinase receptors. The α- and β-receptors have molecular sizes of ~170 and 180 kDa, respectively, after maturation of their carbohydrates. They have five immunoglobulin (Ig)-like domains in their extracellular parts and tyrosine kinase domains intracellularly, which have characteristic inserted sequences without homology to other kinases (Claeson-Welsh et al. 1989, Matsui et al. 1989). The PDGF α receptor gene is localized on chromosome 4q12 close to the genes for the structurally related stem cell factor receptor (Spritz et al. 1994) , whereas β receptor gene is located on chromosome 5 (Yarden et al. 1986) close to the gene for structurally related colony stimulatory factor-1 receptor (Roberts et al. 1988).

PDGF does not only interact with matrix molecules but also with soluble proteins. Like many other cytokines, PDGF binds to α_2-macroglobulin (Lamarre et al. 1991). This interaction, which involves PDGF-BB but not PDGF-AA (Bonner and Osornio-Vargas 1995), regulates the amount of PDGF available for interaction with receptors. Another PDGF binding protein is called PDGF-associated protein (PAP) (Fischer and Schubert 1996). PAP binds PDGF with low affinity and it was found to

enhance the activity of PDGF-AA but depress the activity of PDGF-BB. Moreover, the extracellular part of PDGF α-receptor has been detected in normal human plasma; it is possible that such circulating soluble receptors can compete with cell-associated PDGF receptors for ligand binding (Tiesman and Hart 1993).

4.2. Target cells and cellular effects

PDGF receptors are particularly localized on connective tissue cells (Table 3.). The classical target cells for PDGF, fibroblasts and smooth muscle cells, have both α and β receptors, although generally more β receptors. Other cell types also express both receptors, including kidney mesangial cells, Leydig cells, and certain cells in the central nervous system. There are, however, cells that express only one of the two receptor types. Thus, liver sinusoidal endothelial cells and platelets have only α receptors whereas myoblasts and pericytes have only β receptors (Östman and Heldin 2001). Importantly, the level of PDGF receptor expression is not constant. For instance, the expression β-receptors on connective tissue cells in vivo is low but increases during inflammation (Rubin et al. 1988a) or after explantation into tissue culture (Terraccio et al. 1988).

TABLE 3. Normal cell types expressing PDGF receptors

Cell type	PDGFR-α	PDGFR-β	Reference
Platelets/megakaryocytes	x		Vassbotn et al. 1994, Yang et al. 1997
Macrophages	x	x	Inaba et al. 1993
Myeloid hematopoiteic cells		x	Daynes et. al 1991, DeParseval et al. 1993
T-cells		x	Daynes et. al 1991, DeParseval et al. 1993
Kidney mesangial cells	x	x	Alpers et al. 1992
Capillary endothelial cells		x	Bar et al. 1989, Smits et al. 1989
Pericytes		x	Lindahl et al. 1997
Myoblasts		x	Jin et al. 1990
Fibroblasts	x	x	Heldin and Westermark 1999
Vascular smooth muscle cells	x	x	– " –
Liver sinusoidal endothelial cells	x		– " –
Itoh cells of the liver		x	– " –
Neurons	x	x	– " –
Astrocytes	x		– " –
Schwann cells	x	x	– " –
Retinal pigment epithelial cells	x	x	– " –
Leydig cells	x	x	– " –
Mammary epithelial cells	x	x	– " –

The PDGF α- and β-receptor homo- and heterodimers induce similar, but not identical, cellular effects. In vitro, a selective list of PDGF actions on connective tissue cells includes: migration, proliferation, contraction, inhibition of gap junctional communication and alteration of cellular

metabolic activities including matrix synthesis, cytokine production and lipoprotein uptake (Heldin and Westermark 1999). PDGF also exerts an antiapoptotic effect (Yao and Cooper 1995).

4.3. PDGF-mediated intracellular signal transduction

Ligand-induced dimerization of PDGF receptors activates a number of intracellular signalling pathways, which ultimatey lead to cell growth, changes in cell morphology, and prevention of apoptosis. An extensive cross talk between different signalling pathways and the occurrence of parallel positive and negative signals modulate the responses. Dimerization of the receptor molecules brings the intracellular parts close to each other, thereby allowing autophosphorylation between the two receptors. The autophosphorylation occurs on specific tyrosine residues and has two important consequences: phosphorylation of a conserved tyrosine residue located in the activation loop of the kinase domain leads to an increase of the kinase activity of the receptor and phosphorylation of several tyrosine residues outside the kinase domain produces docking sites for signaling molecules with SH2 domains (Heldin et al. 1998).

Signal transduction pathway is initiated by binding of each one of the SH2 domain-containing molecules. SH2 domains contain ~100 amino acid residues folded in such a way that they recognise phosphorylated tyrosine residues in spesific environments (Pawson and Scott 1997). A large number of SH2 domain-containing signalling molecules have been shown to bind to autophosphyralated α and/or β receptors (Heldin et al. 1998). Some of these molecules are themselves enzymes, e.g. phosphatidylinositol-3'- kinase (PI-3-kinase) and phospholipase C-γ (PLC-γ), the Src family of tyrosine kinases, the tyrosine phosphtaseSHP-2, and the GTPase-activating protein (GAP) for Ras. Other SH2 domains molecules, including Grb2, Grb7, Crk, Nck and Shc lack enzymatic activity and serve as adaptors linking the receptors with downstream effector molecules. Also, Stat molecules bind to PDGF receptors, after which they act as transcription factors and regulate the activity of specific genes (Östman and Heldin 2001). There is an extensive cross talk between different signalling pathways, creating an intracellular signalling network (Fig. 5).

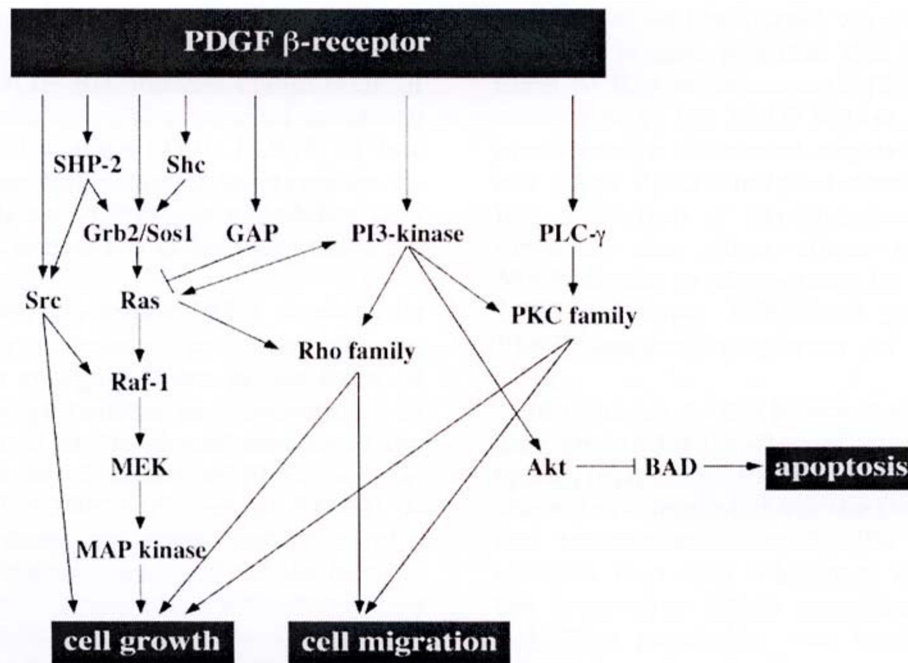

FIGURE 5. The main signalling pathways after PDGFR-β activation which lead to cell growth, migration and anti-apoptosis (Heldin et al. 1998). The figure is reproduced by the permission of the authors and the publisher.

PI3-kinase has been shown to be particularly important for cell motility and antiapoptotic responses. It is essential for PDGF-induced actin reorganization and chemotaxis (Wennström et al. 1994a, b). PLC-γ appears not to be essential for any of the PDGF responses. However, it makes cells more suspectible to migratory responses (Rönnstrand et al. 1999). Activation of Ras, which leads to activation of the MAP kinase cascade, is important for the mitogenic effect of PDGF (Bos 1997). Also the tyrosine kinase Src has been shown to be important for the mitogenic effect of PDGF (Twamley-Stein et al. 1993). In addition, Src may affect cytoskeletal organization and cell morphology via activation of another tyrosine kinase, Abl (Plattner et al. 1999).

Several mechanisms for modulation of signaling via PDGF receptors have been elucidated. MAP kinase, which is activated by Ras, phosphorylates and inactivates Sos, which theryby leads to decreased Ras activation (Porfiri et al. 1996). Another negative-feedback mechanism involves cAMP-dependent protein kinase, which is activated by PDGF through induction of prostaglandin synthesis and activation of adenyl cyclase (Graves et al. 1996). Moreover, angiotensin II has been shown to delay PDGF-BB induced DNA synthesis in vascular smooth muscle cells (Dahlfors et al. 1998).

4.4. In vivo function of PDGF

PDGF-PDGF receptor signaling has critical functions in the ontogeny of connective tissue cells. Knock-out mice studies has revealed this in the mesangial cells of the kidney glomeruli (Levéen et

al. 1994) and in the alveolar smooth muscle cells of the lung (Boström et al. 1996). Defective development of blood vessels (Lindahl et al. 1997) and heart (Levéen et al. 1994) as well as cranial malformations and deficiency of myotome formation (Soriano 1997) have been also described in knock-out mice. To date, reports have published four knock-outs: PDGF-B (Levéen et al. 1994), PDGFR-β (Soriano 1994), PDGF-A (Boström et al. 1996) and PDGFR-α (Soriano 1997). The notion that PDGF and PDGF receptors have important roles during embryonic development is supported by the findings that in each case the mice died during embryogenesis or perinatally.

PDGFs play a central role in normal wound healing. PDGF does not appear to alter the normal sequence of repair, but it increases its rate. It stimulates mitogenicity and chemotaxis of fibroblasts and smooth muscle cells and chemotaxis of neutrophils and macrophages (Heldin and Westermark, 1999). It also stimulates macrophages to produce and secrete other growth factors as well as several matrix molecules, like fibronectin (Blatti et al. 1988), collagen (Canalis 1981), proteoglycans (Schönherr et al. 1991), and hyaluronic acid (Heldin et al. 1989).

PDGF has been shown to have an angiogenic effect (Battegay et al. 1994). The effect is weaker than that of bona fide angiogenic factors of the VEGF or FGF families. However, in specific organs, the effect of PDGF on angiogenesis may be significant. PDGF has also been implicated in the regulation of the tonus of blood vessels (Berk et al. 1986, Sachinidis et al. 1990, Cunningham et al. 1992). Another effect of PDGF that is of importance in the vascular system is its feedback control effect on platelet aggregation (Bryckaert et al. 1989). PDGF has also an important role to maintain the interstitial fluid pressure (Rönnstrand et al.1999).

4.5. PDGF in disease

Because of PDGF's multiplicity of activities, much attention has been also focused on its' involvement in the pathogenesis of various diseases ranging from atherosclerosis to rheumatoid arthritis, liver cirrhosis and cancer.

The finding that PDGF causes transformation and malignant tumors in experimental systems raises the question of whether PDGF is involved as an autocrine growth factor in the development of spontaneous tumors in humans. Many tumors express PDGF and cognate receptors, and in these an autocrine stimulation of tumor cell growth may prevail. In the genesis of glioblastoma and sarcoma an autocrine PDGF receptor activation may be a rate-limiting event (Guha et al. 1995, Smits et al. 1992). In addition to being an autocrine growth factor, PDGF is also involved in paracrine stimulation of stromal cells of certain tumors. Such tumors include mammary carcinoma (Anan et

al. 1996), colorectal cancer (Sundberg et al. 1997), small-cell lung carcinoma (Kawai et al.1997), also the development of myelofibrosis in chronic myelogenous leukemia has been ascribed to PDGF. Novel PDGFs are also expressed in many tumors and tumor cell lines (Li and Eriksson 2003), and PDGF-D is demonstrated to be a potent transforming and angiogenic growth factor (Li et al. 2003).

PDGF has a well-established role in the development of atherosclerosis. While PDGF is expressed at low levels in arteries from healthy adults, its expression is increased in conjunction with the inflammatory-fibroproliferative response that characterizes atherosclerosis (Ross 1993). Especially, PDGF-BB and PDGFR-β seem to be significant in neointima formation (Rutherford et al. 1997, Sirois et al. 1997), whereas PDGF-AA appears to be less important (Hart et al. 1997). In vitro adult rat aortic smooth muscle cells in culture can be induced to proliferate with nanomolar concentrations of PDGF-BB (Mäkelä et al. 1999). Besides its mitogenic activity, PDGF is a chemoattractant for diploid fibroblasts and arterial smooth muscle cells. In addition to direct effects on vascular smooth muscle cell growth and migration, PDGF is capable of inducing in vascular smooth muscle cell in culture an early gene (JE), that encodes a monocyte chemoattractant. The human JE codes a monocyte secretory protein (Taubman et al. 1992, Rollins et al. 1989), and may play a critical role in attracting monocytes to sites of inflammation in atherosclerosis. Recently it was even shown that inhibition of PDGFR-β signalling by red wine flavonoids offers a molecular explanation for the "French paradox" ie. for the phenomenon that moderate wine consumption protects from atherosclerosis (Rosenkranz et al. 2002).

FIGURE 6. The healthy effects of red wine are partly due to PDGFR-β inhibition.

Recent studies have shown that overactivity of PDGF is involevd in the pathogenesis of several glomerular diseases that are characterized by mesangial cell proliferation, including IgA nephropathy, membranoproliferative glomeuronephritis and lupus nephritis (Abboud 1995). Of novel PDGFs PDGF-C is shown to be constitutively expressed in the kidney and specifically up-regulated in mesangial, visceral epithelial, and interstitial cells after injury to these cells in rat (Eitner et al. 2002). PDGF-D is also expressed in developing and mature human kidneys (Changsirikulchai et al. 2002). A role for PDGF in various forms of lung fibrosis is also suggested by observations of PDGF overexpression in patients affected by these diseases, as well as by various lung fibrosis models with increased PDGF expression (Hertz et al. 1992, Harmon et al. 1994, Yi et al. 1996, Ohta et al. 1997 Kallio et al. 1999). PDGF has also been implicated in liver cirrosis (Pinzani et al. 1996). In chronic synovial inflammation, PDGF and PDGF receptors are upregulated (Reuterdahl et al. 1991, Rubin et al. 1988b, Sano et al. 1993), suggesting a role for PDGF in mesenchymal cell proliferation in rheumatoid arthritis. Other fibrotic conditions where PDGF may be involved are palmar fibrosis (Dupuytren's contracture) (Alman et al. 1996), scleroderma (Gay et al. 1989, Klareskog et al. 1990) and myelofibrosis of the bone marrow (Gersuk et al. 1989). Induction of PDGF has also been observed in chronic transplant rejection both in experimental and clinical studies (Fellström et al. 1989, Alpers et al. 1996, Floege et al. 1998, Sihvola et al. 1999, Kallio et al. 1999).

4.6. PDGF and PDGF receptors as drug targets

As overactivity of PDGF is linked to several serious disorders, clinically useful PDGF antagonists are highly warranted. Various types of PDGF antagonists have been developed and have beneficial effects in various animal models; their potential clinical utility is currently evaluated.

Neutralizing polyclonal and monoclonal antibodies against PDGF isoforms, SELEX aptamers, soluble PDGF receptors are antagonists that interfere with the binding of PDGF to its receptors (Östman and Heldin 2001). In experimental models effects of these have been shown in restenosis and glomerulonephritis. Low molecular weight tyrosine kinase inhibitors antagonize PDGF by blocking the down-stream signal transduction of PDGF receptors. They constitute a novel class of drugs with large potential. To date number of selective PDGF receptor kinase inhibitors have been characterized and six compounds -CGP53716 (Buchdunger et al. 1995), STI571 (Buchdunger et al. 1996), Ki6898 (Yagi et al. 1998), RPR101511A (Bilder et al. 1999), AG1296 (Kovalenko et al. 1994) and SU101 (leflunomide) (Shawver et al. 1997) – have demonstrated in vivo efficacy in various animal models. STI571, known also as imatinib, is the most investigated of these compunds. Imatinib, a derivative of 2-phenylaminopyrimidine (Fig 7.), has been shown to inhibit both PDGF α

and β receptors in vitro, it is also cross-reactive with c-Kit and vAbl-Bcr (Buchdunger et al 2000). It is believed to be a competitive antagonist of ATP binding that blocks the ability of PDGFR to transfer phosphate groups from ATP to tyrosine residues on substrate proteins, which in turn interrupts PDGFR-mediated signal transduction. In clinical oncology, this compound has been shwon to be well tolerated and curative to chronic myeloic leukemia and gastrointestinal stromal tumours (Druker et al. 2001, Joensuu et al. 2001). Different methods of gene transfer have also been used to block PDGF signaling in experimental models (Östman and Heldin 2001).

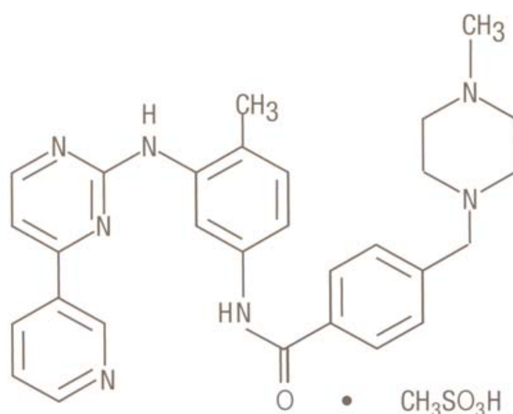

FIGURE 7. Chemical structure of imatinib.

Pharmacological interventions into the generation of PDGF by platelets have also described. Dipyridamole selectively decreased the PDGF levels in human serum, while other antiplatelet drugs, such as aspirin, trapidil, ticlopidine, were found to be inactive (Takehara et al. 1990). In contrast to that report Vissinger et al. have demonstrated that low dose aspirin produced a significant decrease in relase of PDGF from platelets of helathy volunteers (Vissinger et al. 1993). Naftidrofuryl was also shown to decrease the release of PDGF from platelets of healthy adults (Barradas et al. 1994). The same effect was shared by trapidil in platelets from patients affected by vascular inflammation (Bonfardeci et al. 1994).

AIMS OF THE STUDY

The specific aims of this study were:

To study the role of PDGF and its receptors in acute renal allograft rejection and chronic allograft nephropathy in a controlled experimental animal model

To study the effect of acute rejection and cyclosporine-treatment on PDGF induction in this model during the development of chronic allograft nephropathy

To study experimentally the long-term effects of tacrolimus on chronic allograft nephropathy and PDGF expression, and compare tacrolimus to cyclosporine

To study the effect of cyclosporine and tacrolimus on PDGF expression in monocyte-macrophages in vitro

METHODS

1. Kidney transplantations

Specific pathogen-free, inbred male Wistar Furth (WF) AG-B2, $RT1^u$ and Dark Agouti (DA) AG-B4, $RT1^a$ rats (The Laboratory Animal Centre, University of Helsinki, Helsinki, Finland) weighing 300-350 g and of 3-4 months of age were used for transplantations. They received regular rat food (Altromin, Standart Dioet; Chr. Petersen A/S, Ringsted, Denmark) and tap water ad libitum, and were maintained on a 12-h light/dark cycle. The animals received human care in compliance with the *Principles of Laboratory Animal Care* and the *Guide for the Care and Use of Laboratory Animal Resources* published by the National Institutes of Health (NIH publication no. 86-23, revised 1985).

Allogenic kidney transplantations were performed from DA to WF rat strain and syngenic kidney transplantations were done between DA rats using a modified microsurgical technique of Fisher and Lee (Fisher and Lee 1965). The donor kidney was excised, perfused with phospate-buffered saline containing 0.4% 5000 IU/ml heparin, and stored in the same solution at +4ºC until used for transplantation. The kidney was transplanted heterotopically to recipients abdominal aorta and inferior vena cava utilizing end-to-side aortic and vena caval anastomosis under intraperitoneal chloral hydrate anesthesia (240 mg/kg). Ureteral anastomosis was performed end-to-end close to the renal pelvis. Right native kidney was removed during transplantation. Left nephrectomy was performed on day 7 after transplantation. Buprenorphinum (Temgesic; Reckitt & Colman, Hull, England) was used for postoperative pain relief. Total perioperative ischemia time was standardized between 35 and 50 min, beginning with exsanguination and cold perfusion of the graft and ending with revascularization of the graft. The grafts were removed 1, 3, 5 and 7 days after transplantation to study acute rejection and 90 days after transplantation to study chronic allograft nephropathy.

2. Drug regimens

Cyclosporine. CsA (Sandimmun, Novartis, Basel, Switzerland) was dissolved in Intralipid (Kabi Vitrum, Stockholm, Sweden) to final concentrations of 1.5 and 5 mg/ml and administered s.c. once a day or mixed in drinking water (Neoral, Novartis, Basel, Switzerland) to a concentration corresponding the dose 5 mg/kg/d. CsA administration was started at transplantation and continued until the graft was removed, except in the group where the impact of acute rejection episodes on the development of chronic allograft nephropathy was studied. In this group rats were treated with CsA 5 mg/kg/d s.c. for one week and then left untreated until the development of acute rejection. Acute

rejection was diagnosed when serum creatinine value was over 200 μmol/l and treated with CsA 5 mg/kg/d s.c. until the normalization of creatinine value or time of sacrifice. CsA (Sandimmun, Novartis, Basel, Switzerland) was used also for in vitro mononuclear phagocyte experiments.

Tacrolimus. Tac (FK506 SDF formulation, an oral formulation provided by Fujisawa, Munich, Germany) was suspended in aqua to final concentrations of 1.6 and 3.2 mg/ml and administered perorally once a day, starting at transplantation and continued until the graft was removed. Tac (Prograf, Fujisawa, Munich, Germany) was used for in vitro mononuclear phagocyte experiments.

Imatinib. STI571 (Glivec, Novartis, Basel, Switzerland), a protein tyrosine kinase inhibitor selective for PDGF receptors (Buchdunger et al. 2000) was dissolved in aqua to final concentration of 10 mg/kg and administered p.o. once a day, starting at transplantation and continued until the graft was removed. This dose has been shown to be well-tolerated and effective in inhibition of intimal hyperplasia after rat aorta denudation (Savikko et al. 2003).

3. Tests to monitor the clinical course of renal transplanted rats

Serum creatinine and concentrations of studied immunosuppressive drugs were measured once a week until the rats were sacrificed. Serum creatinine levels were measured using method by Jaffe (Whelton et al. 1994). House made reagents according to the reagent kit by JT Baker: "Creatinine Jaffe-Modified 3837" for quantitative determination of creatinine in biological fluids (JT Baker Chemicals BV, Deventer, Holland) were used and the samples were analyzed by Cobas Mira Analyzer (Roche). Whole blood CsA levels were measured using radioimmunoassay (Sandimmun-Kit, Novartis, Basel, Switzerland), and whole blood tacrolimus levels were measured using a microparticle enzyme immunoassay (IMx; Abbott Laboratories, Abbott Park, IL).

4. Histological stainings

The kidney grafts were bisected horizontally and fixed in 3% paraformaldehyde for 24h. The specimens were sectioned 2 μm in thickness and stained with Mayer's hematoxylin-eosin, Masson's trichrome, diastase-periodic acid-Schiff, methenamine silver periodic acid-Schiff and Unna-Pappenheim stains. To analyze acute rejection changes 22 histologic features (I) were scored blindly using an arbitrary scale from 0 to 3, 0 indicating no pathological alterations, 1 mild changes, 2 moderate changes and 3 extreme changes. The chronic changes in the allografts harvested 90 days after transplantation were scored according to Chronic Allograft Damage Index (CADI). The

CADI is expressed as a single numerical figure to show the intensity of chronic changes in the transplant (Isoniemi et al. 1992, Solez et al. 1993). The CADI value is a sum of six parameters scored from 0-3 including interstitial inflammation and fibrosis, tubular atrophy, glomerular mesangial matrix increase, glomerular sclerosis and arterial intimal proliferation.

5. Immunohistological stainings

For immunohistochemistry renal samples were embedded in O.C.T. (Tissue-Tek; Miles Inc., Elkhart, IN), snap-frozen in liquid nitrogen, and stored at –70 °C. Four µm thick sections were cut in series on glass slides. Before staining, the slides were air-dried at least for 2 h and fixed in acetone at -20 °C for 10 min.

To demonstrate the expression and localization of PDGF-AA and -BB as well as PDGFR-α and -β the samples were immunostained using Vectastain Elite ABC Kit (Vector Laboratories Inc., Burlinghame, CA) (I-IV). The same staining method was used to rule out the existence of c-Kit and v-Abl in kidney grafts (III). For immunostaining the specimens were incubated with primary antibodies at optimal dilution at +4 °C for 15 h. The primary antibodies were diluted in TRIS-NaCl -buffer, pH 7.40. With intervening washes in TRIS-NaCl -buffer, the specimens were incubated with biotinylated goat anti-rabbit absorbed antibodies in TRIS-NaCl -buffer at room temperature (RT) for 30 min; avidin-biotinylated horseradish complex in TRIS-NaCl -buffer at RT for 30 min; and the reaction was revealed by chromogen 3-amino-9-ethylcarbazole containing 0.1% hydrogen peroxidase, yielding a red-brown reaction product. The slides were counterstained with Mayer's hemalum and mounted (Aquamount; BDH Ltd., Poole, England). Polyclonal rabbit IgG antibodies to PDGF-AA (2 µg/ml, sc-7958), -BB (2 µg/ml, sc-7878), PDGFR-α (1 µg/ml, sc-338) and -β (1 µg/ml, sc-339), c-Kit (2 µg/mol, sc-168) and v-Abl (1 µg/mol, sc-131) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Recombinant human homodimers for PDGF-AA and -BB and control peptides for PDGFR-α, PDGFR-β, c-Kit and v-Abl to test the specificity of polyclonal antibodies were obtained from the same source. As negative controls were used samples stained with the same protocol but without the primary antibodies. For polyclonal antibodies control peptides were used as negative controls, too. Ten-fold amount of control peptide than the used amount of polyclonal antibody was mixed with the antibody and incubated for 12 h before staining. After incubation, the control samples were stained with these mixes using the same protocol as described above. Appropriate tumor tissue samples were used as positive controls for c-Kit and v-Abl antibodies.

To demonstrate the localization of macrophages and T-cells (III), the samples were immunostained using a 3-layer indirect immunoperoxidase technique (von Willebarnd et al. 1985). The primary monoclonal mouse antibodies used were ED3 (Serotec Ltd, Oxford, UK,), rat CD4 and rat CD8 (BD PharMingen). Peroxidase-conjugated rabbit anti-mouse Ig (DAKO A/S, Denmark) and peroxidase-conjugated goat anti-rabbit IgG (Caltag Laboratories, Burlington, CA) were used sequentially.

Immunohistochemical analysis was done in a blind review. The intensity of the PDGF ligand and receptor stainings was scored from 0 to 3 as follows: 0, no visible staining; 1, cells with faint staining; 2, moderate intensity with multifocal staining; and 3, intense diffuse staining. The morphology of positively stained cells was also analysed. CD4, CD8 and ED3 positively stained cells were counted in three visual fields from the renal cortex at maginification x400, and the mean number of positive cells per field of vision was calculated.

6. Monocyte-macrophage –cell experiments

The U937 cell line (kindly provided by Dr K. Nilsson, University of Uppsala, Sweden) was used as an in vitro model for studying the effect of CsA and Tac on PDGF induction in monocyte-macrophages (Sundström and Nilsson 1976). Phorbol ester 12-O-tetradecanoyl-phorbol-13-acetate (TPA) was used to induce the cells to differentiate into macrohages. The U937 cells were kept at logaritmic growth in RPMI-1640 containing 5% normal human serum, 100 IU/ml penicillin, 100 µg/ml streptomycin and 1% glutamine (all from GIBCO BRL, Paisley, England). The cells were grown at 37ºC in humidified atmosphere with 5% CO_2. Cell growth was monitored by microscoping the cells by an inverted microscope and counting the cells in a cell chamber. Viability of the cells was determined by trypan blue exclusion.

To study the effect of CsA and Tac on PDGF induction and secretion in monocyte-macrophages, the U937 cells were treated together with 10^{-8} M TPA either with CsA or Tac at concentrations $1x10^{-7}$ and $1x10^{-8}$ g/ml corresponding to the clinical blood trough levels 100 ng/ml and 10ng/ml of these drugs respectively. CsA (Sandimmun, Novartis, Basel, Switzerland) and Tac (Prograf, Fujisawa, Munich, Germany) were diluted in 100% ethanol to concentrations such that addition of 0.01% (v/v) ethanol in each well produced the desired final concentration. TPA-treated cells were used as controls. After 3 days culture, the conditioned media was removed for ELISA assay of PDGF-AB and PDGF-BB levels. Each experiment was performed in triplicate.

7. PDGF measurements

Conditioned media obtained from cultures of CsA- and Tac-treated differentiated U937 cells were assayed for levels of human PDGF-AB and PDGF-BB utilizing the enzyme-linked immunosorbent assay method. Commercial kits for this purpose were purchased from R&D Systems Inc. (Minneapolis, MN, USA). The assays were performed as recommended by the manufacturer, except that the assay was sensitized by using 9:1 diluted samples and 120 min incubations with substrate solutions instead of of 1:1 dilutions and 30 min incubations. The detection limit was 1 pg/ml both for PDGF-AB and PDGF-BB. The results of the PDGF assays were expressed as pg of PDGF/ml of conditioned medium.

8. [^{3}H]Thymidine incorporation studies

The proliferating U937 cells were seeded at approximately 0,5-1,0 x 10^6 cells/ml in 96-well plastic dishes, and exposed to different concentrations of CsA and Tac as described earlier. For the measurement of DNA synthesis after 24h, 48h or 72 h exposure to either CsA or Tac, 100 µCi/ml of [^{3}H]thymidine (925GBq/mmol, 25Ci/mmol, Amersham) was added to each well for the last 4h of culture. The cells were then harvested and transferred on paper filters using a cell harvester (Skatron Combi, Skatron, Norway) and ^{3}H-radioactivity was measured in liquid scintillation counter. The results are presented as percent of control ± SEM from three individual experiments done with three parallels.

9. Morphologic analysis of U937 cells

Morphology of the U937 cells was analyzed after exposure to CsA and Tac. The cells were cytocentrifuged on cytopreps. Slides were fixed and stained with May-Grunwald-Giemsa stain, and cells were examined under a microscope at 40 x magnification to determine the morphologic features of monocyte-macrophage differentiation. The cells were classified as monoblasts, monocytes or macrophages according to their morphologic features.

10. Statistical methods

Each kidney transplantation group consisted of 3-5 rats, and the cell experiments were repeated at least 3 times. The results are expressed as mean ± SEM, and a probability of < 0.05 was accepted as significant. The significance between groups was determined by analysis of variance, Fisher's PLSD (StatView 4.1, Abacus Concepts, Inc., Berkley, CA).

RESULTS

1. Experimental rat kidney transplantation (I-IV and unpublished results)

Clinical course. CsA or Tac were used as immunosuppression at doses adjusted to be clinically relevant. Blood trough levels of these drugs were measured weekly until the grafts were harvested. Both drugs were well-tolerated in WF rats and no recipients were lost before the end of the follow-up period. No adverse effects were seen in transplanted WF rats based on appearance, behaviour or weight loss. In contrast DA rats with syngenic kidney transplants treated with CsA had severe adverse effects if treated with similar doses than WF rats. They died within 3 weeks due to weight loss. CsA blood trough levels appeared to be much higher in these animals. When CsA doses within this group were adjusted to reach approximately the same blood trough levels as in WF rats, the animals behaved normally and no abnormal weight loss was seen, no higher doses could be used because of side effects.

Because the left native kidney was left in situ there were no signs of renal failure and creatitine levels were normal in acute rejection study groups at the end of the investigated time period. Serum creatinine levels were controlled weekly in all transplanted rats during the 90 day follow-up. In CsA-treated allografts serum creatinine values were clearly higher than in syngenic control animals. In low-dose CsA-treated group creatinine values were slightly lower than in high-dose group (II). In animals that had acute rejection episodes during the development of chronic allograft nephropathy the average creatinine levels were constantly higher than in low- or high-dose CsA-treated animals (II). Acute rejection was diagnosed when serum creatinine value was over 200 μmol/l and treated with CsA until the normalization of creatinine value or time of sacrifice. These animals had 0-2 CsA-treated acute rejection episodes. In Tac-treated animals the average creatinine values were somewhat higher than in syngenic control animals. There was no significant difference between the values of low- and high-dose groups in Tac-treated animals. There was a trend towards higher creatinine values in CsA-treated animals compared to Tac-treated animals (IV).

Acute rejection. In the syngenic grafts, no signs of acute rejection were observed. In contrast, the nontreated allografts developed severe, mixed interstitial-vascular type, acute rejection within 7 days (I, III, IV). In the non-treated allografts, endothelialitis with subendothelial mononuclear inflammatory cell infiltration and endothelial swelling developed during the progression of acute rejection and also intimal thickening was seen. Later, also capillary thrombosis was observed. The number of graft infiltrating inflammatory cells increased constantly during the observation period. Interstitial and perivascular inflammatory cell infiltrates consisted mainly of mononuclear

inflammatory cells, initially lymphocytes and later also macrophages. With the progression to necrosis also neutrophils were observed. Similar pattern was seen in glomerular and tubular inflammation. CsA-treatment (1.5mg/kg/d) ameliorated histological changes of acute rejection (I, III, IV), especially diffuse interstitial inflammation, compared to untreated allografts. However, acute rejection progressed despite of treatment and the overall extent of rejection reached slight to moderate level within 7 days. Tac ameliorated markedly the development of acute rejection, very few inflammatory changes were seen in both Tac-treated groups (1.6mg or 3.2 mg/kg/d) (IV). Inflammation was somewhat lower in high-dose than in low-dose Tac treated allografts. At day 7 there was no statistically significant difference between histological alterations between syngenic and Tac-treated kidney transplants.

Chronic allograft nephropathy. In syngenic grafts no histological signs of chronic allograft nephropathy were seen, CADI 0.3±0.2. CADI values of CsA- and Tac-treated animals are summrized in Table 4.

TABLE 4. Histological changes analyzed by CADI score in CsA- and Tac-treated allografts (II, IV)

	Syn	CsA 1.5mg/kg	CsA 5mg/kg	Tac 1.6mg/kg	Tac 3.2mg/kg
Inflammation	0.1±0.0	1.8±0.4	1.1±0.2	0.7±0.3	0.4±0.1
Fibrosis	0.1±0.0	1.3±0.4	1.6±0.2	0.3±0.2	0.1±0.1
Glom matrix increase	0.2±0.2	2.0±0.2	1.4±0.2	1.1±0.2	1.2±0.1
Intimal proliferation	0.2±0.2	0.9±0.2	0.8±0.2	0.6±0.1	0.9±0.2
CADI	0.3±0.2	6.5±1.3	5.4±0.6	3.3±0.6	2.6±0.4

Values are mean±SEM

High-dose CsA-treatment ameliorated inflammation compared to low-dose CsA-treatment, although it failed to inhibit the development of chronic changes. More fibrosis was even seen in high-dose CsA-treated grafts. Glomerular mesangial matrix increase was significant in both CsA-treated groups compared to syngenic grafts. At day 90 only mild chronic changes were seen at both Tac doses. Inflammation and fibrosis were somewhat lower in high-dose than in low-dose Tac-treated group, these histological changes were significantly lower in both Tac-treated groups compared to CsA-treated allografts ($p<0.05$). Acute rejection episodes during the development of chronic allograft nephropathy increased chronic histological changes in the allografts (II), especially arterial intimal proliferation, $p<0.05$ compared CsA-treated control allografts. CADI value for these grafts with acute rejection episodes was 8.0 ±1.2. Glomerular sclerosis was not seen

in any allograft group (II-IV). Tubular atrophy was also almost nonexistent at the end of the investigated time period in all allografts, although significant atrophy in tubuli was seen earlier during the development of chronic rejection in groups with late acute rejections (II). In addition, no histological signs associated to drug induced-nephrotoxicity were seen in the investigated allografts.

2. Platelet-derived growth factor is induced already early in acute renal allograft rejection (I, IV)

In the syngenic controls PDGF ligand and receptor expression was almost nonexistent 1-7 days after transplantation. PDGF-AA and -BB as well as PDGFR-α and -β were significantly induced in capillary endothelial cells and infiltrating macrophages during the development of acute rejection both in the non-treated allografts and in the CsA-treated allografts, which showed moderate acute rejection changes. In the non-treated allografts PDGF ligands and receptors were expressed until the allografts developed necrosis, whereas in the CsA-treated allografts the expression remained high during the time period investigated. PDGF-AA was found to be induced more rapidly than PDGF-BB, although at the end of the study period the expression of both ligands was nearly at the same level. Similarly, PDGFR-α was induced more strongly than -β at the onset of acute rejection in interstitial inflammatory cells and capillaries, whereas at the end of the study the expression of both receptors was at equal level. PDGF ligands and receptors were also expressed in arterioles and arteries in the non-treated and the CsA-treated allografts. Some expression was also seen in glomerular leukocytes. In arterioles, arteries and glomerular leukocytes PDGF-AA was expressed more strongly than PDGF-BB, whereas the expression level of PDGFR-β was higher than that of PDGFR-α.

Very mild acute rejection was demonstrated in Tac-treated allografts at early days after transplantation at both doses studied. Tac with both doses studied inhibited also significantly PDGF ligand and receptor expression compared to CsA-treated allografts ($p<0.05$). Some expression was seen in the few infiltrating macrophages, capillary endothelial cells and glomerular leukocytes of Tac-treated allografts early after transplantation. The expression was highest at day 5, by day 7 it had started to decrease, whereas in CsA-treated allografts expression remained high at day 7.

3. The effect of acute rejection on PDGF induction during the development of chronic allograft nephropathy (II)

Chronic allograft nephropathy was associated with induction of all PDGF ligands and receptors (II). Acute rejection episodes during the development of chronic allograft nephropathy increased PDGF expression even more. The expression of PDGF ligands and receptors was already induced in late acute rejection in inflammatory cells, capillary endothelium and arterial smooth muscle cells and it increased further during the development of chronic rejection. At the end of the investigation significantly more PDGFR-β expression was seen in arterial smooth muscle cells in allografts that had acute rejection episodes during the follow-up compared to stable allografts. In atrophied and degenerated tubuli of late acute rejection groups PDGF ligand and receptor expression was almost nonexistent whereas induced expression was seen in intact areas of tubuli in chronic rejection at the end of the 90 day follow-up.

4. Platelet-derived growth factor receptor tyrosine kinase inhibition prevents chronic allograft nephropathy (III)

Clinical course. Both CsA and imatinib were well tolerated, no adverse effects were seen in imatinib-treated animals and no recipients were lost before the end of the follow-up period. There were no statistical differencies with CsA blood trough levels between the group of imatinib-treated allografts and CsA-treated control allografts. Creatinine values of imatinib-treated animals were markedly lower compared to CsA-treated control animals, they were at the same level as in animals with syngenic kidney transplants.

Histology. Syngenic control grafts showed no histological signs of acute rejection or chronic allograft nephropathy, CADI value was 0.8±0.2. Imatinib did not affect the development of acute rejection. Similar mild to moderate acute rejection findings were seen in basal CsA-immunosuppressed control allografts as well as in allografts treated with imatinib+CsA. Allografts treated only with imatinib developed massive acute rejection, similar to untreated control allografts. In CsA-treated control allografts intense chronic changes were seen, CADI 7.8 ±1.4. Imatinib inhibited the development of chronic allograft nephropathy almost totally, only very few histological changes were seen. CADI value was 1.3±0.4, and all histological parameters scored were significantly lower compared to CsA-treated control allografts, $p<0.05$. No significant differencies were seen in histological changes between imatinib-treated and syngenic grafts.

Immunohistochemistry. Alhough imatinib selectively inhibits PDGFR tyrosine kinases, it has also cross-reactivity with Abl and c-Kit tyrosine kinases. However, no v-Abl expression was seen in any of the studied kidney grafts. Very slight c-Kit expression was seen in tubuli of all studied grafts, as has also been seen in normal kidneys (Natali et al. 1992). Moderate to intense expression of PDGF ligands and receptors was seen in basal CsA-immunosuppressed control allografts. Almost no PDGF ligand and receptor expression was seen in syngenic grafts either early after transplantation or at the end of study. Some expression of PDGF receptors was induced early after transplantation in imatinib-treated allografts, whereas at the end of the study the PDFG ligand and receptor expression was almost nonexistent. Imatinib inhibited also significantly the infiltration of macrophages and CD4+ T-cells into the grafts both early after transplantation as well as during the long-term follow-up.

5. The impact of cyclosporine and tacrolimus on PDGF induction during the development of chronic allograft nephropathy (II, IV and unpublished data)

In syngenic grafts the expression of PDGF ligands and receptors remained almost nonexistent during the 90 day follow-up. Chronic allograft nephropathy in both low- and high-dose CsA-treated animals was associated with induction of all PDGF ligands and receptors, $p<0.05$ compared to syngenic controls (II). In CsA-treated allografts moderate to intense PDGF expression was seen in interstitial inflammatory cells, capillary endothelium, arterial smooth muscle cells, glomerular leukocytes and basement membranes of tubuli. Both ligands and receptors were seen in inflammatory cells, capillaries, glomeruli and tubuli, whereas more expression of PDGF-BB and PDGFR-β was seen in arteries. High-dose CsA-treatment induced PDGF receptors even more in inflammatory cells, capillary endothelium, arterial smooth muscle cells and tubuli compared to low-dose CsA-treatment.

In Tac-treated animals at both doses studied PDGF expression 90 days after transplantation was significantly decreased compared to CsA-treated animals. The expression was located mainly in the interstitial leukocytes, some expression was also seen in arterial smooth muscle cells and glomerular leukocytes. PDGF ligands and receptors were expressed very similarly in the few infiltrating leukocytes, whereas more PDGF-BB and PDGFR-β was seen in arterial smooth muscle cells and glomerular leukocytes.

As high-dose CsA-treatment increased fibrosis and PDGF expression compared to low-dose CsA-treatment in renal allografts, the effect of CsA on syngenic transplants was also studied to

investigate whether alloimmune response is needed for the development of fibrosis and PDGF induction or does CsA itself cause these changes in renal transplants (unpublished data). In syngenic grafts treated with CsA marked histological changes compared to untreated syngenic grafts were seen (Fig. 8). Especially fibrosis and glomerular mesangial matrix accumulation were significantly increased compared to untreated syngenic grafts (* p<0.05). In addition, PDGF expression was significantly induced in CsA-treated syngenic grafts compared to untreated syngenic grafts (Table 5.). No difference was, however, seen in kidney function between untreated and CsA-treated syngenic grafts based on creatinine levels.

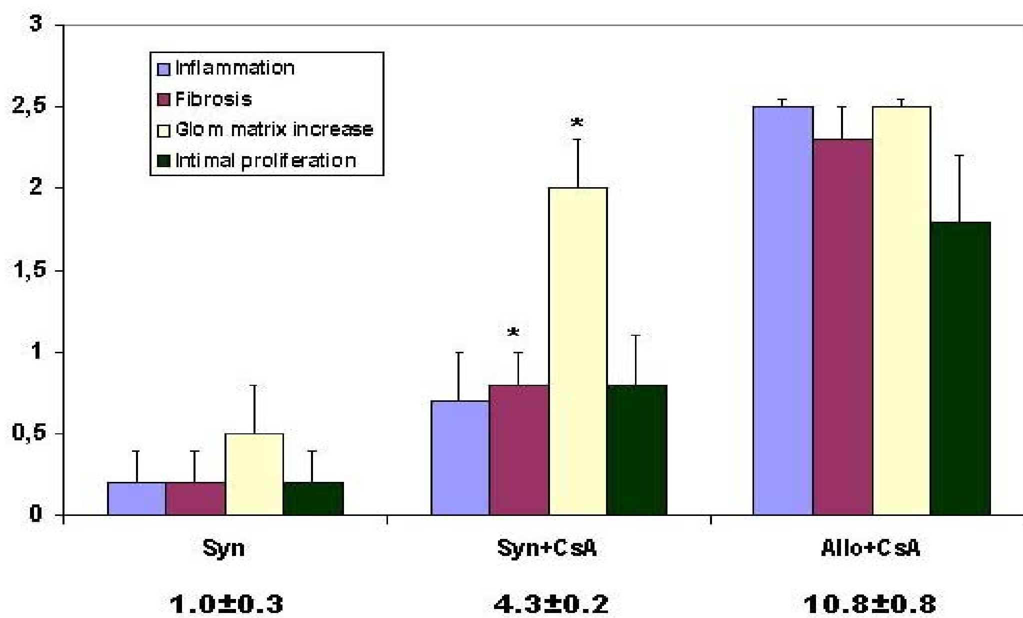

FIGURE 8. Histological analysis 90 days after transplantation. Values are CADI±SEM. The bars show the each parameter scored from 0 to 3. No tubular atrophy or glomerular sclerosis was seen in any of the studied grafts.

TABLE 5. PDGF expression was significantly induced in CsA-treated syngenic grafts compared to untreated syngenic grafts in the following histological areas (x=p<0.05)

	leukocytes	artSMC	capillaries	tubuli	glomeruli
PDGF-AA	x	x		x	x
PDGF-BB			x	x	x
PDGFR-α		x			x
PDGFR-β	x	x	x	x	x

6. The effect of cyclosporine and tacrolimus on PDGF expression in monocyte-macrophages (IV)

Monoblastic cell line U937 was used to study the effects of CsA and Tac on PDGF induction during the monocytic differentiation (IV). The U937 cells were induced with 10^{-7} M TPA to differentiate into macrophages in three days. At day 3 approximately half of the cells showed macrophage phenotype. Higher TPA concentration could not be used because of the cytotoxic effects on the cells (Savikko and von Willebrand 2001). Tac and CsA at different concentrations were added simultaneously with TPA to the cultures. At day 3 PDGF-AB and –BB levels were measured from conditioned culture media by enzyme-linked immunosorbent assay.

[^{3}H]Thymidine incorporation studies showed no toxicities for used concentrations of Tac or CsA in U937 monocyte-macrophage cultures. Neither CsA nor Tac inhibited TPA-induced differentiation of U937 cells into macrophages.

PDGF-AB levels in untreated control cells ranged from undetectable to 1.5 pg/ml, and PDGF-BB levels from undetectable to 5.5 pg/ml. At clinically relevant 10^{-7} g/ml concentration CsA induced significantly both PDGF-AB and –BB levels in macrophage cultures compared to untreated control cells and 10^{-7} g/ml and 10^{-8} g/ml Tac-treated cells ($p<0.05$). PDGF levels were 8.5±1.3 (PDGF-AB), and 13.8±3.8 (PDGF-BB). PDGF levels in CsA 10^{-8} g/ml- and Tac-treated cells remained at the same level as was seen in the untreated controls.

DISCUSSION

Chronic allograft nephropathy still remains the major unsolved problem in clinical kidney transplantation. The annual rate of graft loss due to chronic allograft nephropathy is 3-5% (Hariharan et al. 2000). Currently there is no treatment available for preventing it. Although acute rejection is demonstrated to be the single most important risk factor for the subsequent chronic allograft nephropathy, the molecular mechanisms leading to chronic changes are largely unknown. The better knowledge of basic biology in rejection processes will give new insights for future therapeutical interventions and prevention of chronic allograft nephropathy.

1. Experimental rat kidney transplantation as a model for chronic allograft nephropathy

Chronic allograft nephropathy requires months to years to develop in man, and therefore it is almost impossible to study the pathological molecular mechanisms in detail. However, experimental models give invaluable insight for the problems seen in clinical kidney transplantation. The experimental transplantation between DA and WF rats provides a strongly histoincompatible model for studying the development of acute and chronic rejection. Without immunosuppression the grafts are lost to massive acute rejection and necrosis in 7 days (I). Discontinuation of immunosuppression later on will also easily result in late acute rejection (II). The clinically relevant concentrations of CsA and Tac are well-tolerated in WF rats (II-IV). Compared to well-established Fisher to Lewis model of rat kidney transplantation this model provides a possibility to investigate the long-term effects of immunosuppressive agents as they are administered daily. In Fisher to Lewis model immunosuppression is not needed after initial 10 day immunosuppression (White and Hildeman 1968, Diamond et al. 1992).

The immunosuppression in this rat model using either CsA or Tac seems to produce histological alterations similar to clinical kidney transplantation. As both native kidneys are removed in our model the measurement of serum creatinine values offers a useful tool for monitoring kidney function. Based on these values kidney function in this model also seems to reflect clinical situation. In addition, the variation both in histological and functional values measured between individual rats has proved to be quite low. Although our experimental rat model is a highly simplified system compared to clinical setting, the results obtained in this model can be consired relevant and they can direct further studies with primates before clinical application.

2. Early induction of PDGF in acute rejection may start the molecular cascades leading to chronic allograft nephropathy

It is hypothesized that acute rejection could cause the primary injury leading to induction of reparative mechanisms resulting in interstitial fibrosis and mesenchymal cell proliferation. However, the exact molecular pathways between acute and chronic rejection are yet largely unknown. In the present study, we demonstrate that PDGF-AA and -BB and their receptors α and β are induced in acute rat renal allograft rejection (I). A prominent finding was the induction of PDGF ligands and receptors in infiltrating macrophages, capillary endothelial cells and arterioles simultaneously with histological changes of acute rejection already three days after transplantation.

The histological findings of the study show the presence of combined acute cellular and vascular rejection. CsA ameliorated the severity of acute cellular rejection, but failed to inhibit it. The expression of PDGF ligands and receptors was induced in the affected areas indicating the connection between histological changes and PDGF expression. In contrast Tac inhibited acute rejection almost totally and also PDGF expression remained nearly nonexistent. This further confirms the link between acute rejection and PDGF induction.

The expression of PDGF ligands and receptors in capillary endothelial cells was a significant finding. The strongly induced expression of both PDGF-AA and -BB as well as of PDGFR-α and -β in infiltrating macrophages and capillary endothelial cells indicates that PDGF is involved in signalling and inducing mesenchymal cell proliferation and fibrosis already at acute rejection, which may also contribute to the development of subsequent chronic rejection. Antibodies could play an important role as mediators in this process. Previously the presence of donor specific antibodies have been associated both to acute rejection and development of subsequent chronic rejection (Oluwole et al. 1989, Suciu-Foca et al. 1991). The precise mechanism of this antibody-mediated graft injury is not known, but it is hypothesized that growth factors could be involved in this process (Ciubotariu et al. 1998). In our study donor-specific antibodies were not examined. However, our data could also support the previous findings. Antibodies are thought to injure graft endothelium, and thus induced PDGF expression in capillary endothelium and infiltrating macrophages could be a mediator in antibody-associated acute and chronic rejection.

Intimal proliferation in muscular arteries is a major finding in chronic rejection (Häyry et al. 1993) and PDGF is known to be a very potent stimulus for smooth muscle cell migration and proliferation (Fingerle et al. 1989, Jackson et al. 1993). Previously it has been demonstrated that PDGFR-β is upregulated in arteries in chronic renal allograft rejection (Rubin et al. 1988a, Fellström et al.

1989). The upregulated expression of PDGFR-β in arteries, together with continuous release of PDGF from activated macrophages, platelets, and possibly other cell types, is suggested to constitute a basis for pathological tissue proliferation in chronic vascular inflammation (Rubin et al. 1988a). It is also known that the PDGF-A chain peptide is expressed by both intimal and medial smooth muscle cells in arteries with chronic rejection, whereas the PDGF-B chain is mainly located in infiltrating monocytes (Alpers et al. 1996). Here we demonstrate similar findings in acute rejection. PDGF-AA was shown to be expressed more intensively than -BB in arterioles and arteries during the development of acute rejection. Moreover, the expression of PDGF-AA remained high in arterioles and arteries in CsA-treated allografts at the end of the investigation. We could also demonstrate induction of PDGFR-β in arteries during the development of acute rejection. Induction of PDGF ligands and receptors in small capillaries and arteriolar smooth muscle cells suggests the onset of vascular changes already in acute rejection. Upregulation of PDGFR-β in arterial smooth muscle cells in acute rejection could thus be linked to the development of subsequent vascular lesions in chronic rejection.

Histological changes of glomeruli are also associated with chronic rejection (Isoniemi et al. 1992). PDGF ligand and receptor induction was seen in glomerular leukocytes in our study. Previous studies have shown that PDGF is a strong mitogen for glomerular mesangial cells (Abboud 1995). The PDGF receptor expression in glomerular leukocytes in acute rejection could thus be associated also to later glomerular mesangial matrix increase and sclerosis in chronic rejection. The thrombosis of glomerular capillaries associated to renal rejections could be a source of PDGF (von Willebrand et al. 1985).

In conclusion, our results demonstrate that PDGF ligands and receptors are induced already in acute rat renal allograft rejection. This suggests that PDGF is involved at the onset of acute rejection and the early induction of PDGF may start the molecular cascades that link acute rejection to chronic rejection.

3. Acute rejection episodes enhance PDGF expresion during the development of chronic allograft nephropathy

Interstitial inflammation and fibrosis, vascular changes, glomerular mesangial matrix increase and tubular atrophy are major histogical alterations in chronic rejection of renal transplants (Isoniemi et al. 1992). PDGF is suggested to be major mitogen for mesenchymal cell proliferation in chronic allograft bephropathy (Fellström et al. 1989, Alpers et al. 1996, Floege et al. 1998). Although acute rejection is known to be the single most important risk factor for the subsequent development of chronic rejection, very little is known about the molecular mechanisms operating between acute and chronic rejection. In our study moderate to intense chronic changes were seen in all CsA-treated allograft groups (II). Acute rejection episodes enhanced the developmet of chronic changes which is consistent with earlier experimental and clinical studies (Yilmaz and Häyry 1993, Troppman et al. 1995). In addition, we report here that acute rejection episodes increased the expression of PDGF ligands and receptors in the development of chronic rat renal allograft rejection.

We have demonstrated that PDGF is induced already at acute rejection (I). This induction may start the molecular mechanisms leading to chronic rejection. The results of the present study confirm our earlier data. Chronic allograft nephropathy was firmly linked to induction of all PDGF ligands and receptors at the end of the study. The expression of PDGF ligands and receptors was already induced in late acute rejection groups in inflammatory cells, capillary endothelium and arterial smooth muscle cells and it further increased during the development of chronic rejection. Arterial intimal proliferation was significantly increased in allografts with acute rejection episodes compared to syngenic and CsA-treated allografts. Important finding was that PDGFR-β was also induced more in arterial smooth muscle cells of these allografts. This is in accordance with previous studies suggesting the role of PDGF as a major mitogen in allograft arteriosclerosis (Fellström et al. 1989, Alpers et al. 1996, Floege et al. 1998). Although the increased expression of PDGFR-β was associated with enhanced histological changes in the allograft group with acute rejection episodes, the statistical significance compared to the CsA-treated allograft groups remained nonexistent mainly due to the heterogenicity of the allograft group with acute rejection episodes. However, our results give strong evidence for the role of acute rejection as an inducer of vascular damage and enhanced PDGF-β expression in arterial smooth muscle cells.

Taken together, it seems likely that PDGF has a role in the process leading from acute to chronic rejection. The present study demonstrates that PDGF overexpression is strongly associated to

chronic allograft nephropathy and that acute rejection episodes induce even more PDGF and its receptors indicating a link between acute rejection and subsequent development of chronic rejection. Based on the present findings the inhibition of action of PDGF could be a potential intervention site in preventing the development of chronic renal allograft damage.

4. Imatinib is a promising candidate drug for prevention of chronic allograft nephropathy

The development of chronic allograft nephropathy is a multifactorial process including both immunological and nonimmunologic factors (Halloran et al. 1999, Paul 2000). However, the exact mechanisms leading to chronic allograft nephropathy are largely unknown. Overexpression of PDGF ligands and receptors has been shown to associate with acute rejection and chronic allograft nephropathy (Fellström 1989, Alpers et al. 1996, Floege 1998, I, II), although the definite role of PDGF in these pathological events is not known.

In the present study we demostrate that blocking the PDGF receptor signalling with a PDGF receptor tyrosine kinase inhibitor imatinib prevented the development of chronic allograft nephropathy in rat. Nearly normal kidney function was also seen in imatinib-treated allografts during long-term follow-up.

Imatinib, formerly also known as STI571, was used in this study to investigate the role of PDGF in the development of chronic allograft nephropathy. Imatinib has been shown to inhibit both PDGF α and β receptors in vitro (Buchdunger 2000). It is also cross-reactive with c-Kit (CD117) and vAbl-Bcr. However neither c-Kit nor vAbl-Bcr was associated with chronic allograft nephropathy in the studied kidney grafts whereas PDGF ligand and receptor expression was highly induced in CsA-treated control allografts with chronic rejection. This indicates that the action of imatinib was focused directly to PDGF receptors in the graft. Very recently it has been demonstrated that proliferating intimal cells in transplant arteriosclerosis originate from recipient bone marrow (Shimizu et al. 2001, Sata et al. 2002). c-Kit may have a role in the migration of these cells from bone marrow into the graft vessels.

Imatinib did not affect the overall development of acute rejection, although it inhibited the infiltration of macrophages and CD4+ T-cells into the grafts compared to CsA-treated control allografts. Interestingly, however, it could prevent chronic allograft nephropathy despite of mild to moderate acute rejection seen in imatinib-treated allografts early after transplantation. The

decreased infiltration of macrophages at acute rejection may be an important feature in the prevention of subsequent development of chronic allograft nephropathy, as macrophages in the graft are shown to be a predisposing factor to the development of chronic changes (von Willebrand 1992, Croker et al. 1996). Based on this it seems likely that imatinib can very effectively inhibit the infiltration of monocyte-macrophages into the graft and thus prevent the repair processes in the graft after acute rejection induced injury by inhibiting PDGF receptors; also interfering with c-Kit signalling might play a role in this process. However, PDGF receptors were induced in the studied grafts already early after transplantation whereas no c-Kit expression was associated to rejection processes indicating that PDGF inhibition is the main mechanism in preventing mesenchymal cell proliferation in the pathogenesis of chronic allograft nephropathy.

Fibrosis, intimal proliferation and histological changes of glomeruli are typical for chronic allograft nephropathy (Isoniemi et al. 1992). In imatinib-treated allografts of the current study the development of fibrosis was significantly decreased compared to control allografts suggesting that PDGF has a very important role as a fibrogenic growth factor. Fibrotic lesions were as minimal as in syngenic controls. Here we show also that almost no intimal proliferation was seen in imatinib-treated allografts whereas moderate neointima formation was seen in CsA-treated control allografts. Our current data confirms the earlier hypothesis of PDGF as a major mitogen for arterial smooth muscle cell proliferation in renal allografts. PDGF is a strong mitogen for glomerular mesangial cells (Abboud 1995). PDGF ligand and receptor expression was demonstrated in glomerular leukocytes of CsA-treated control allografts in this study together with glomerular changes. Imatinib inhibited markedly glomerular mesangial matrix increase in the glomeruli compared to control allografts indicating that the inhibition of PDGF prevents also the histological changes of glomeruli.

The important finding of the study was not only the nearly normal histological findings but also the good function of the imatinib-treated allografts. Creatinine values of imatinib-treated allografts were at the same level as in syngenic control grafts during the long-term follow-up. This encourages for the use of imatinib in clinical kidney transplantation.

In conclusion, our data demonstrate that imatinib prevents chronic allograft nephropathy almost completely indicating that PDGF plays a very important role in its pathogenesis. Based on our findings imatinib could also be a potential intervention in preventing chronic allograft nephropathy in clinical kidney transplantation.

5. Tacrolimus seems to be more safe and effective in the long-run than cyclosporine

During the 20 years when CsA has been used as immunosuppressive medication, chronic allograft nephropathy still has remained the major reason for late allograft dysfunction (Paul and Fellström 1992, Cecka 1999). Thus, it seems likely that there are mechanisms involved in the development of chronic rejection that CsA can not inhibit. There is also both experimental and clinical evidence that the accelerated form of transplant arteriosclerosis may be linked to the administration of CsA, even within therapeutic levels (Mennander et al. 1991, Bohman et al 1991). CsA has been found to induce PDGF both in vivo and in vitro (Nares et al. 1996, Iacopino et al. 1997). There is also evidence that fibrosis of renal transplants caused by CsA-treatment may also be due to induced expression of other growth factors like TGF-β_1 (Khanna et al. 1997, Shin et al. 1998) and IGF-1 (Johnson et al. 1999).

Currently Tac is used successfully as a de novo agent for acute rejection prophylaxis and for rescue therapy in clinical solid organ transplantation. The first multicenter studies have shown that Tac has diminished the incidence and severity of acute rejection compared to CsA (Pirsch et al. 1997, Mayer et al. 1997, Margreiter 2002), also some improvement is seen in the incidence of chronic rejection and in long-term allograft survival some years after transplantation (Mayer 1999, Vincenti et al. 2002). Still, the effect of this newer immunosuppressant on the development of chronic changes is unknown in the long run. Tac is a calcineurin inhibitor like CsA and nephrotoxicity effects similar to CsA have been documented using this drug (de Mattos et al. 2000). However, the drugs are structurally different and thus likely to have also functional differencies.

We have investigated the effects of different doses of CsA and Tac on chronic allograft nephropathy and PDGF induction (II, IV). To further investigate the possible differencies in the actions of CsA and Tac we used mononuclear phagocyte cell cultures to study the effect of these calcineurin inhibitors on PDGF induction and secretion during monocytic differentiation (IV). Also the effect of CsA on syngenic transplants was studied to investigate whether alloimmune response is needed for the development of fibrosis and PDGF induction or does CsA itself cause these in renal transplants (unpublished results).

Important finding was that high-dose CsA-treatment could not inhibit the development of chronic changes, although it ameliorated the extent of inflammation compared to low-dose CsA-treatment (II). At the end of the investigated time period the chronic changes scored by CADI were almost equal in both CsA-treated allograft groups. In accordance with previous studies high-dose CsA-treatment induced even more fibrosis than low-dose CsA-treatment (Ruiz et al. 1988, Remuzzi and

Perico 1995, Bennett et al. 1996). Chronic changes of both low- and high-dose CsA-treated allografts were strongly associated with induction of PDGF ligands and receptors. PDGF ligands were distributed very similarly in both low- and high-dose CsA-treated allografts, whereas PDGF receptors were more expressed in inflammatory cells, capillary endothelium, arteriolar smooth muscle cells and tubuli of high-dose CsA-treated allografts. In addition in syngenic grafts treated with CsA marked histological changes compared to untreated syngenic grafts were seen, especially fibrosis and glomerular mesangial matrix accumulation (unpublished results). Also PDGF expression was significantly induced in these grafts compared to untreated syngenic grafts in glomeruli, capillaries, tubuli, arterial smooth muscle cells and interstitial leukocytes. Our findings suggest that PDGF has an important role in mediating the development of chronic changes in renal transplants and that the accelerated form of chronic rejection associated to the CsA-administration may be linked to the enhanced induction of PDGF receptors.

The induced expression of PDGF and TGF-β have been previously also associated to the nephrotoxicity of CsA in experimental rat models (Shehata et al. 1994, Shehata et al. 1995). However, the doses used in those studies were significantly higher compared to our doses. The doses used in our study represent CsA-blood levels currently used in clinical kidney transplantation, the other being somewhat suboptimal and the other being somewhat above the optimal levels. In addition no histological signs associated to CsA-nephrotoxicity (Yilmaz et al. 1992) were seen in the investigated allografts.

We also demonstrate here that Tac significantly inhibits the induction of PDGF expression and the development of rejection changes compared to CsA both early after transplantation as well as during long-term follow-up of rat renal allografts (IV). The used Tac doses were chosen to represent approximatley similar clinical situation as was with used CsA-doses (II). The low-dose was suboptimal and the high-dose was at the optimal levels. Tac has shown to be a very potent immunosuppressant in clinical transplantation, even more potent than CsA (Polsker and Foster 2000). In our study almost no histological signs of acute rejection were seen in either with low- or high-dose Tac-treated allografts some days after transplantation whereas in CsA-treated allografts moderate to intense acute rejection was seen. This indicates that Tac is a more effective immunosuppressant compared to CsA. This is also consistent with clinical data showing the improved results with Tac compared to CsA in treating acute rejection (Pirsch et al. 1997, Mayer et al. 1997, Margreiter 2002). Interestingly, also the chronic allograft changes were significantly milder in Tac-treated allografts compared to CsA-treated allografts. Significantly less inflammation and fibrosis was seen in the both Tac-treated allograft groups compared to CsA-treated allografts at

the end of the follow-up period. Only very mild chronic changes were seen in the both Tac-treated allograft groups. Also the creatinine levels of Tac-treated animals were somewhat lower than those of CsA-treated animals.

The more normal histological findings were also strongly associated with significantly less induced expression of PDGF ligands and receptors both early after transplantation as well as during long-term follow-up in Tac-treated allografts compared to CsA-treated grafts. In addition to our finding that PDGF inhibition prevents chronic allograft nephropathy almost completely (III), this marked association between inhibited PDGF expression and improved long-term results shown here further confirms the importance of PDGF as an important mediator in the pathological process leading to chronic allograft nephropathy. Our findings also further confirm the earlier data of acute rejection as a strong inducer of chronic changes (Yilmaz et al 1993, Troppman et al. 1995, II). Effective prevention of acute rejection seems to be an important issue in improving the long-term results of kidney grafts.

Although Tac is a calcineurin inhibitor like CsA it seems to be more safe in the long run than CsA. It is documented that Tac is a less potent inducer of TGF-β in human kidney allografts than CsA (Mohamed et al. 2000). Although TGF-β has been demonstrated to be more induced in transplant recipients with Tac-nephrotoxicity compared to CsA-nephrotoxicity (Khanna et al. 2002), there are many studies, however, suggesting that Tac is less fibrogenic than CsA (Mohamed et al. 2000, Bicknell et al. 2000, Baboolai et al. 2002, Waller et al. 2002). In addition, the study by Khanna et al. may be biased by the fact that nephrotoxicity may always be highly associated with TGF-β regardless of whatever the treatment has been before. In our study PDGF ligand and receptor expression during long-term follow-up of renal allografts was significantly lower in Tac-treated allografts compared to CsA-treated allografts. Some expression was seen in the interstitial leukocytes, arterial smooth muscle cells and glomerular leukocytes in Tac-treated allografts indicating that some mesenchymal proliferation is taking place also in these allografts in the long run. However, based on our present findings it seems likely that Tac and CsA have in part different functional mechanisms.

Monocytic cell infiltrates are typical for acute vascular rejection, which is often resistant to immunosuppressive therapy (von Willebrand et al. 1992), and not dominated by the usual T-cell driven mechanisms. Humoral mechanisms might be involved in this rejection type (von Willebrand et al. 1992). Monocyte-macrophages play also a central role in chronic rejection (Croker et al. 1996). They are the major celltypes synthesizing growth factors. The release of PDGF from

macrophages is believed to be instrumental during connective tissue overgrowth (Pierce et al. 1991). In addition PDGF is suggested to act as an chemoattractant for monocyte-macrophages (Rollins et al. 1989, Siegbahn et al 1990, Taubman et al. 1992). The induction of PDGF ligands and receptors in interstitial mononuclear leukocytes demonstrated already early in acute rejection may thus be an important finding (I). Previously we have shown that PDGF may have an autocrine effect on monocytic differentiation and maturation in vitro (Savikko et al. 2001). PDGF induction could also be involved in the development of acute vascular rejection by having effect on monocytic differentiation and maturation. The finding that CsA could not inhibit the PDGF ligand and receptor expression in our model with mixed interstitial-vascular type acute rejection may partly explain why acute vascular rejection is often resistant to immunosuppressive medication. Increased PDGF induction in CsA-treated allografts could explain the pronounced accumulation of monocytes into CsA-treated allografts and the development of acute vascular rejection. In addition CsA has been shown to induce PDGF in cultured human macrophages (Iacopino et al. 1997). Interestingly, we could show here similar PDGF induction in cultured U937 macrophages treated at clinically relevant concentration of CsA, whereas no induction was seen in Tac-treated cells. This suggests that the ability of CsA to induce growth factors and cytokines is probably independent of its calcineurin inhibitory activity. This finding may also explain the decreased incidence of severe, steroid-resistant acute rejection in Tac-treated kidney allograft recipients compared to CsA-treatment (Margreiter 2002). This type of rejection is shown to be a very high risk factor for the development of subsequent chronic rejection (van Saase et al. 1995). The decreased incidence of acute vascular rejection in Tac-treated renal allografts may also predict better long-term outcome.

Taken together, our results demonstrate that Tac and CsA have different effects on PDGF induction on rat renal allografts and macrophages in vitro. PDGF induction and rejection changes were inhibited in renal allografts of Tac-treated rats both early after transplantation as well as during long-term follow-up. This may be due to the more potent immunosuppressive effect of Tac and its milder direct growth factor inductory effect compared to CsA. Our study suggests that Tac may be more safe in the long run than CsA and thus have a beneficial effect on later outcome of the kidney graft.

6. Conclusions

The data of this thesis suggest that PDGF plays an important role both in acute and chronic renal allograft rejection.

We have demonstrated that PDGF ligands and receptors are induced already at acute rat renal allograft rejection (I). This early induction of PDGF in acute rejection may start the molecular cascades that link acute rejection to subsequent development of chronic allograft nephropathy. We have also shown that acute rejection episodes during the development of chronic allograft nephropathy induce even more PDGF and chronic changes (II). These findings encourage for better treatment of acute rejection episodes in clinical transplantation to prevent further development of chronic allograft nephropathy.

We have also shown that blocking PDGF receptor signalling with a PDGF receptor tyrosine kinase inhibitor, imatinib (STI571), prevented the development of chronic allograft nephropathy in rat (III). Nearly normal kidney function was seen in imatinib-treated allografts over a long-term follow-up indicating that PDGF plays a very important role in the pathogenesis of chronic allograft nephropathy. Based on our findings imatinib could also be a potential intervention in preventing chronic allograft nephropathy in clinical kidney transplantation.

We have also demonstrated that high-dose Cyclosporine A (CsA) –treatment increases PDGF ligand and receptor expression in the graft compared to low-dose CsA-treatment during the development of chronic allograft nephropathy indicating a poor outcome of the kidney graft (II). In addition CsA increases PDGF in syngenic kidney grafts suggesting that it may directly accelerate the development of chronic changes via growth factor induction. We have also shown that tacrolimus significantly inhibits PDGF induction and the development of rejection changes both early after transplantation as well as during long-term follow-up of rat renal allografts compared to CsA (IV). In addition, we have demonstrated that CsA induces PDGF secretion in macrophages in vitro, whereas Tac does not. (IV). This suggests that tacrolimus may be more safe than CsA in clinical kidney transplantation in the long run and thus have a beneficial effect on later outcome of the kidney graft.

In conclusion, this study helps to understand the molecular mechanisms in rejection processes and provides a new candidate drug for preventing chronic allograft nephropathy in clinical kidney transplantation.

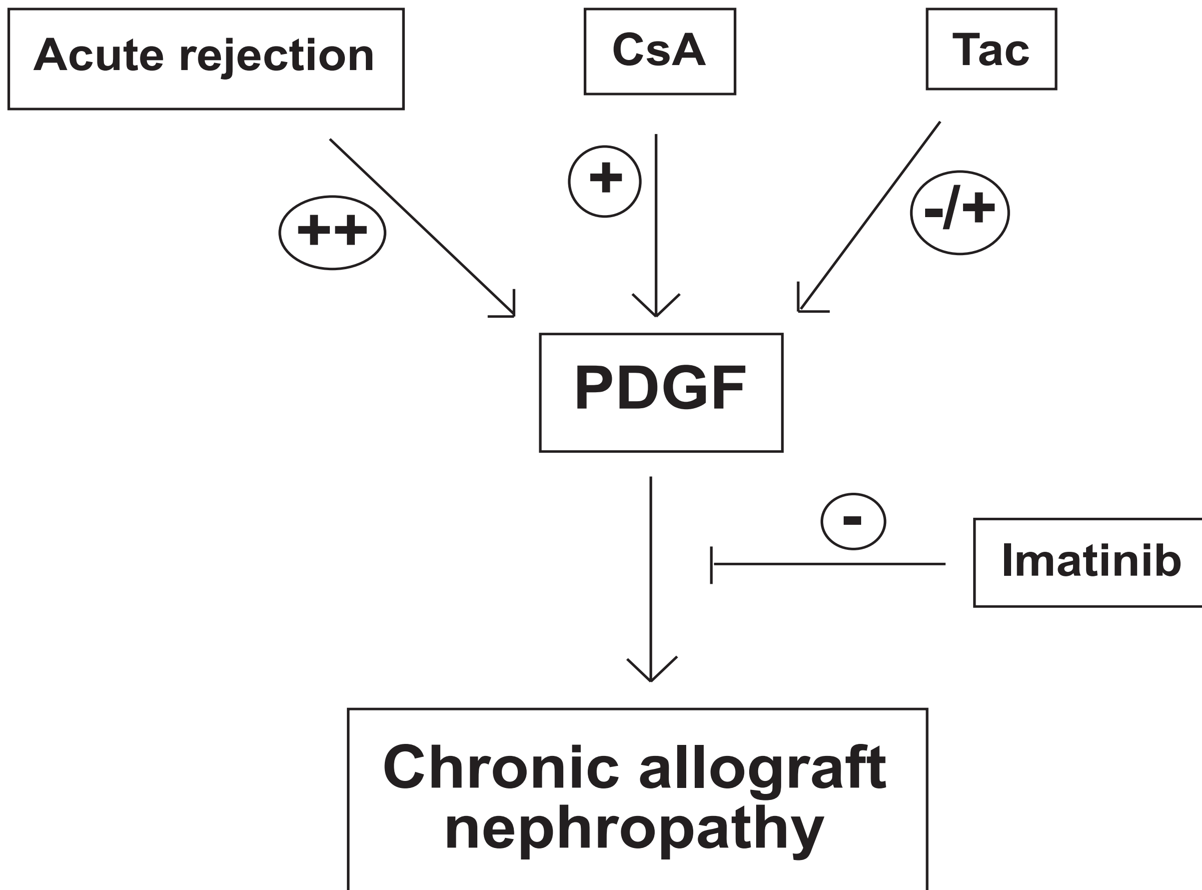

FIGURE 9. This thesis demonstrates that PDGF plays a significant role in the development of CAN. Acute rejection episodes increase the expression of PDGF during the development of CAN. CsA also seems to induce PDGF expression during this pathological process whereas Tac is likely to be more beneficial in the long run by inhibiting or at least inducing less PDGF compared to CsA. Imatinib by blocking PDGF receptor signaling inhibits the development of CAN almost completely and could thus be a potential intervention in preventing CAN in clinical kidney transplantation.

SUMMARY

Chronic allograft nephropathy is the major problem in clinical kidney transplantation. Although cyclosporine has been succesfully used to prevent acute rejections since 1980's, the annual rate of graft loss due to chronic allograft nephropathy is still 3-5%. There is also evidence that the accelerated form of transplant arteriosclerosis and fibrosis may be linked to administration of cyclosporine. Chronic allograft nephropathy is an irreversible fibrotizing process leading eventually to the loss of the graft, and currently there is no treatment available for preventing or treating it. Its development is a multifactorial process including both immunological and nonimmunologic factors. However, the exact mechanisms leading to chronic allograft nephropathy are largely unknown.

Acute rejection is the single most important risk factor for the development of subsequent chronic allograft nephropathy. It is hypothesized that acute rejection could cause the primary injury leading to induction of reparative mechanisms resulting in fibrosis and mesenchymal cell proliferation. PDGF is suggested to be a major mesenchymal mitogen in the development of chronic allograft nephropathy. However, its definite role and importance in the rejection mechanisms is unknown but it can be significant both in acute rejection as a mediator which starts the rejection process and also in chronic allograft nephropathy as a mediator that regulates the inflammatory cascades leading to fibrosis and transplant arteriosclerosis.

The aim of this study was to investigate the role of PDGF in acute and chronic renal allograft rejection and the links between acute and subsequent chronic rejection in an experimental kidney transplantation model.

The data of this dissertation suggest that PDGF plays an important role both in acute and chronic renal allograft rejection. PDGF ligands and receptors were induced already at acute rat renal allograft rejection (I). This early induction of PDGF in acute rejection may start the molecular cascades that link acute rejection to subsequent development of chronic allograft nephropathy. Acute rejection episodes during the development of chronic allograft nephropathy induced even more PDGF and chronic changes (II). These findings encourage for better treatment of acute rejection episodes in clinical transplantation to prevent further development of chronic allograft nephropathy.

Blocking PDGF receptor signalling with a PDGF receptor tyrosine kinase inhibitor, imatinib (STI571), prevented the development of chronic allograft nephropathy in rat (III). Nearly normal kidney function was seen in imatinib-treated allografts over long-term follow-up indicating that PDGF plays a very important role in the pathogenesis of chronic allograft nephropathy. Based on this imatinib could also be a potential intervention in preventing chronic allograft nephropathy in clinical kidney transplantation.

High-dose cyclosporine –treatment increased PDGF ligand and receptor expression in the graft compared to low-dose cyclosporine-treatment during the development of chronic allograft nephropathy indicating a poor outcome of the kidney graft (II). In addition, cyclosporine increased PDGF in syngenic kidney grafts suggesting that it may directly accelerate the development of chronic changes via growth factor induction. Tacrolimus significantly inhibited PDGF induction and the development of rejection changes both early after transplantation as well as during long-term follow-up of rat renal allografts compared to cyclosporine (IV). In addition, cyclosporine induced PDGF secretion in macrophages in vitro, whereas Tac did not. (IV). This suggests that tacrolimus may be more safe than cyclosporine in clinical kidney transplantation in the long run and thus have a beneficial effect on later outcome of the kidney graft.

Taken together, this study helps to understand the molecular mechanisms in rejection processes and provides a new candidate drug for preventing chronic allograft nephropathy in clinical kidney transplantation.

YHTEENVETO (FINNISH SUMMARY)

Krooninen hyljintä on edelleen merkittävin ongelma munuaissiirtopotilailla. Vaikka syklosporiini on vähentänyt huomattavasti akuuttien hyljintöjen määrää 1980-luvulta lähtien, krooniseen hyljintään menetetään edelleen vuosittain 3-5% siirrännäisistä. On myös viitteitä, että syklosporiini saattaa kiihdyttää kroonista hyljintää. Krooniselle hyljinnälle on tyypillistä siirrännäisen vähitellen kehittyvä fibroosi sekä aterosklerosoituminen. Tällä hetkellä ei ole olemassa tehokasta hoitoa kroonisen hyljinnän estämiseksi. Sekä immunologiset että ei-immunologiset syyt johtavat kroonisen hyljinnän kehittymiseen. Kuitenkin krooniseen hyljintään johtavat molekyylimekanismit ovat edelleen suurelta osin tuntemattomia.

Akuutti hyljintä on merkittävin yksittäinen riskitekijä myöhemmin kehittyvälle krooniselle hyljinnälle. On arvioitu, että akuutti hyljintä aiheuttaa kudosvaurion, jonka käynnistämät korjausmekanismit johtavat fibroosiin ja mesenkymaalisten solujen kiihtyneeseen jakautumiseen. Verihiutaleperäisen kasvutekijän (PDGF) on ehdotettu olevan tärkeä solujen jakautumista kiihdyttävä tekijä kroonisessa hyljinnässä. Sen merkitys akuutissa ja kroonisessa hyljinnässä on kuitenkin edelleen epäselvä, vaikka sillä saattaa olla merkittävä osuus sekä akuutin hyljinnän laukaisevena tekijänä että krooniseen hyljintään johtavien solutoimintojen välittäjänä munuaissiirrännäisissä.

Tämän väitöskirjan tavoitteena oli selvittää verihiutaleperäisen kasvutekijän merkitystä akuutissa ja kroonisessa hyljinnässä kokeellisessa munuaissiirtomallissa. Lisäksi tavoitteena oli selvittää akuutin ja kroonisen hyljinnän välisiä yhteyksiä.

Tämä väitöskirjatyö osoittaa, että verihiutaleperäinen kasvutekijä on merkittävä sekä akuutissa että kroonisessa munuaissiirrännäisen hyljinnässä. PDGF:n ja sen reseptoreiden ilmentyminen lisääntyi heti hyljinnän alkupäivinä, millä saattaa olla merkitystä akuutin hyljinnän synnyssä sekä yhteys akuutin ja myöhemmin kehittyvän kroonisen hyljinnän välillä (I). Akuutit hyljinnät lisäsivät edelleen PDGF:n ja sen reseptoreiden ilmentymistä kroonisen rejektion kehittymisessä (II). Nämä löydökset kannustavat akuuttien rejektioiden tehokkaasen hoitoon kliinisessä munuaissiirrossa.

PDGF-reseptorin tyrosiinikinaasia estävä molekyyli, imatinib (STI571), esti munuaissiirrännäisen kroonisen hyljinnän kehittymisen lähes täysin (III). Lähes normaali munuaistoiminta todettiin imatinibia saaneilla munuaissiirtoeläimillä pitkäaikaisseurannassa. Tämä osoittaa, ettö PDGF:llä

on tärkeä osuus kroonisen hyljinnän kehittymisessä. Näihin tuloksiin perustuen imatinibi voi olla myös tehokas lääke kroonisen hyljinnän estämisessä kliinisessä munuaissiirrossa.

Takrolimus esti PDGF:n ja sen reseptoreiden ilmentymistä sekä akuuttien että kroonisten hyljintämuutosten kehittymistä kokeellisessa munuaissiirtomallissa, kun taas syklosporiini lisäsi sekä kasvutekijöiden ilmentymistä että kroonisten hyljintämuutosten syntymistä (II, IV). Lisäksi syklosporiini kiihdytti PDGF ilmentymistä ja erittymistä monosyytti-makrofagisoluviljelmissä, kun taas takrolimuksella ei ollut tätä vaikutusta (IV). Nämä tulokset viittaavat siihen, että takrolimus olisi turvallisempi lääke kuin syklosporiini munuaissiirtopotilaiden pitkäaikaishoidossa.

Tämä väitöskirjatutkimus auttaa ymmärtämään akuutin ja kroonisen hyljinnän molekyylitason syntymekanismeja sekä tarjoaa uuden mahdollisen lääkeaineen kroonisen hyljinnän estämiseen munuaissiirtopotilailla.

SAMMANDRAG (SWEDISH SUMMARY)

Kronisk rejektion är fortfarande ett betydande problem vid klinisk njurtransplantation. Trots att cyklosporin har avsevärt minskat insidensen av akut rejektion jämfört med början av 1980 talet, går årligen 3-5% av njurtransplantaten förlorade i kronisk rejektion. Det finns också tecken på att cycklosporin kan stimulera kronisk rejektion. Aterosklerotiska åderförändringar och fibros, som utvecklas långsamt, är typiska för kronisk rejektion. I dag finns det ännu ingen behandling för att hindra kronisk rejektion. Både immunologiska och ej-immunologiska faktorer är delaktiga i utvecklingen av kronisk rejektion. Molekylära mekanismer i kronisk rejektion är dock dåligt kända.

Akut rejektion är den största enskilda riskfaktorn för uppkomsten av kronisk rejektion. Man har spekulerat i att akut rejektion förorsakar en vävnadsskada som påbörjar reparationsmekanismer vilka sedan leder till fibros och proliferation av mesenkymala celler. Trombocytväxtfaktorn har antagits vara en viktig mitogen för mesenkymala celler i kronisk rejektion. Dess roll i rejektionsmekanismerna är dock dåligt känd, men den kan vara betydande både i akut rejektion som initierande signal, och också i kronisk rejektion kan den reglera olika inflammationskaskader som leder till arterioskleros och fibros.

Avsikten med denna doktorsavhandling har varit att i detalj utforska rollen av trombocyt-växtfaktorn och dess receptorer i akut och kronisk rejektion vid njurtransplantation med försöksdjurmodell. Också sammanhanget mellan akut och kronisk rejektion har utforskats.

Den här avhandlingen visar att trombocytväxtfaktorn har en viktig roll i uppkomsten av akut och kronisk rejektion vid njurtransplantation. Expressionen av trombocytväxtfaktorn och dess receptorer var induserad redan i akut rejektion och också under fasen mellan akut och kronisk rejektion (I, II). Dessa resultat visar att det är viktigt att sköta akuta rejektioner effektivt vid klinisk njurtransplantation.

Genom att förhindra effekten av trombocytväxtfaktorn med en specifik molekyl, imatinib, kunde man förhindra utvecklingen av kronisk rejektion i råttnjurtransplantationsmodellen (III). Nästan normal njurfunktion kunde konstateras hos de imatinib-behandlade djuren. Detta visar att tromcytväxtfaktorn spelar en viktig roll vid utvecklingen av kronisk rejektion. På grund av dessa resultat kan imatinib vara en potentiell terapiform också vid njurtransplantation hos människan.

Cyklosporin-behandling ökade expressionen av trombocytväxtfaktorn och dess receptorer under utvecklingen av kronisk rejektion, när takrolimus förhindrade både rejektionsförändringar och PDGF expressionen under långtids uppföljning (II, IV). Vidare, cyklosporin ökade också expressionen av trombocytväxtfaktorn och dess receptorer i monocyt-makrofag cellodlingsexperiment, men takrolimus ökade inte (IV). Dessa resultat visar att takrolimus kan vara effektivare och tryggare än cyklosporin också vid njurtransplantation hos människan.

Denna doktorsavhandling kartlägger molekylära mekanismer i akut och kronisk rejektion vid njurtransplantation och erbjuder ett nytt potentiellt läkemedel för att hindra utvecklingen av kronisk rejektion hos människan.

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