CHRONIC REJECTION
IN THE AORTA TRANSPLANTATION MODEL

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CHRONISCHE AFSTOTING

IN HET AORTA TRANSPLANTATIE MODEL

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1.1 CLINICAL ASPECTS OF CHRONIC REJECTION

Since the first successful heart transplantation in man in 1967, cardiac transplantation has become an accepted form of therapy for certain types of end-stage heart diseases.[1] After transplantation of a cadaveric heart graft, the immune system of the recipient will come in contact with donor cells. As clinical heart transplantation is an allogeneic type of grafting (genetically non-identical members of the same species), an immune response, which may result in graft rejection, will be inevitable.

Of the foreign antigens that may be recognized by the immune system of the recipient, the major histocompatibility complex (MHC), in man the human leucocyte antigen (HLA) system, is the most important.[2-4] In short, the HLA system consists of a group of closely linked genes, located on the short arm of chromosome-6 and divided in three regions, which encode class I and class II cell surface glycoproteins and several components of the complement system respectively. Class I is expressed on the surfaces of all nucleated cells. Class II is mainly expressed on antigen presenting cells (e.g. macrophages, dendritic cells, and B cells). After transplantation, T cell receptors of recipient CD4+ cells are able to recognize HLA class II, resulting in activation of type 1 and type 2 helper (TH1 and TH2) cells.[5] The activated TH1 cell start to preferentially synthesize and release interleukin-2 (IL-2) and interferon-gamma (IFN-γ). IL-2 stimulates CD8+ T cells to develop into mature cytotoxic effector cells. Binding of these cells to donor HLA class I may result in graft cell lysis. IFN-γ is responsible for the activation of macrophages, which are believed to be cytotoxic to graft cells. TH2 cells preferentially secrete IL-4, IL-6 and IL-10. These interleukins are growth and differentiation factors for B cells. Activation of B cells may result in maturation into plasma cells with allo-specific antibody production.

Clinically, the two most important types of rejection that may occur after transplantation are acute and chronic rejection. Acute rejection is characterized by the infiltration of the graft by recipient mononuclear cells with or without myocyte damage, edema, vasculitis and hemorrhage.[6] This type of rejection may lead, if not adequately treated, to early graft loss. However, the one-year graft survival rate of heart grafts has improved considerably over the past two decades, from around 45% in 1975 to around 85% at the present time.[7] A major factor contributing to this improvement has been the use of cyclosporine (CsA) in the

prevention of acute rejection.[8] With prolonged graft survival, chronic rejection has evoked as a late complication of heart transplantation.[9] The key characteristic of chronic heart rejection is concentric intimal thickening at the entire length of the coronary vessels. This form of coronary artery disease reduces cardiac perfusion and may result in myocardial infarction, development of congestive heart failure, or ventricular arrhythmias leading to sudden death.[10,11] Various terms for chronic rejection are used in literature. These include graft arteriosclerosis, transplant vasculopathy, graft vessel disease, accelerated transplant arteriosclerosis, allograft arteriosclerosis, chronic transplant dysfunction and allograft vasculopathy.

Chronic heart graft rejection is found in as many of 50% of patients who survive more than 5 years post-transplantation.[9,12] This has become the predominant cause of cardiac failure after the initial 6 to 12 months post-transplantation.

Chronic rejection is not only restricted to heart transplants, but has also been described in kidney, liver, lung, pancreas and bowel transplants. In these grafts also, chronic rejection is characterized by pathological factors such as transplant vasculopathy and fibrosis, together with a progressive loss of functions.[13-18]

The presence of chronic rejection in hearts is difficult to detect clinically because for patients with denervated heart grafts it is impossible to experience anginal pain.[19] The first sign of clinically important graft arteriosclerosis is usually electrocardiographic evidence of myocardial infarction. Coronary arterial lesions diagnostic of chronic rejection are rarely present in endomyocardial biopsy, but chronic rejection should be suggested if foci of myocardial infarction are evident.[20] Prior to the development of clinical signs or symptoms of chronic rejection, the principal methods to demonstrate the occurrence of coronary obliteration are coronary angiography or intravascular ultrasound.[21-22]

Contrary to the progress made in the treatment of acute rejection, less is known about the pathogenesis and treatment of chronic rejection. There is still no treatment to prevent graft arteriosclerosis and retransplantation remains the only effective therapy.[23]

1.2 THE PATHOGENESIS OF CHRONIC REJECTION

Introduction

Based on morphologic and functional similarities between the vessel wall lesions of chronic rejection and "naturally occurring" atherosclerosis, Ross proposed in the mid-1970s the "response-to injury" hypothesis to explain its pathophysiology.[24] The primary lesion is, according to this hypothesis, loss of vascular endothelial cell lining, followed by platelet adhesion to the denuded vessel wall. The subsequent release of platelet products like platelet-derived growth factor (PDGF) would stimulate vascular smooth muscle cells (VSMC) to proliferate and migrate into the intima. More recent studies however, showed that the endothelium may remain intact while the vessel wall undergoes arteriosclerotic transformation.[25] In addition, inhibition of platelet function by aspirin or dipyridamol did not reduce graft arteriosclerosis.[26,27] Therefore, the pathophysiology of chronic rejection can not be explained by the hypothesis of Ross.

Alloantigen-dependent factors in chronic rejection

In clinical transplantation it has become clear that MHC class I and II molecules on donor cells provide the primary stimulus for the specific host immune response. That MHC may be involved in chronic rejection, is suggested by histopathological studies of heart allografts, which revealed a correlation between MHC class II antigen expression and graft arteriosclerosis.[28-30]

In addition, graft survival studies have shown that MHC matching is correlated with the incidence of chronic rejection. In these studies, long-term graft survival appeared to be strongly correlated with the degree of histocompatibility between transplant recipient and donor.[31-35] Kidney transplants from HLA identical sibling donors have a half-life of more than 20 years compared with less than 10 years for grafts from non-blood related donors and 7-8 years for completely mismatched cadaveric grafts.[34] It is presently unclear whether the matching effect is a direct effect or whether it results from a decreased incidence of acute rejection episodes as acute rejection episodes correlate strongly with chronic rejection.[35-37] Chronic rejection has been proposed to be the result of recurrent acute rejection episodes.[38]

Acute rejection episodes could persist as a chronic, subclinically smoldering process after treatment with immunosuppressive drugs. Therefore, chronic rejection may be the result of ineffective suppression of acute rejection. This hypothesis in human transplantation is supported by experimental studies. In rat heart and kidney transplantation models, a strong correlation was observed between chronic rejection and the incidence of acute rejection episodes.[39-42]

Although few data are available about cytokines in chronic rejection, cytokines are believed to be involved in the process leading to graft arteriosclerosis. There is some evidence that PDGF, which likely has a role in normally occurring atherosclerosis, plays a similar role in chronic rejection. PDGF was first isolated from platelets but it is produced and secreted by a variety of other cells including monocytes and macrophages[43], endothelial cells[44], and VSMC[45]. In vitro, PDGF has a mitogenic effect on VSMC, but it also stimulates collagen and elastin synthesis and attracts neutrophils and monocytes.[46-48] Various cytokines like TGF-β, IL-1, TNF-α and PDGF itself can induce expression of PDGF mRNA and increased release of PDGF-like protein, suggesting that PDGF is a common pathway in cell growth and proliferation.[48,49] Besides PDGF, other growth promoting cytokines which may be involved in chronic rejection are IFN-γ, IL-1, TNF-α, TGF-β, endothelins and many others.[43,50-54]

Humoral immunity has long been considered to be of importance in chronic vascular rejection.[55,56] Clinical-pathological studies have shown a correlation between anti-MHC class I antibodies, anti-endothelial cell antibodies and chronic vascular rejection.[57-60] The hypothesis that antibodies may be involved in chronic rejection has also been enforced by animal studies. In mice and rabbits donor-specific antibodies have been demonstrated to provoke intimal proliferation.[61,62] Another indication might be the upregulation of the immunoglobulin J chain mRNA in arteriosclerotic lesions.[63] The J chain is needed for the formation of IgM and IgA molecules and suggests the presence of IgM- or IgA-producing plasma cells in such grafts. Immunohistochemical studies of human heart transplants with rejection-associated arteriosclerosis have shown CD4+ and CD8+ lymphocytes and MHC class II positive macrophages in the graft vessel intima.[28] Only few differences are found between infiltrating cell populations of acutely rejecting, chronically rejecting or well-functioning allografts.[64-66] The cellular infiltrate both in acutely and chronically rejecting grafts, consists primarily of T lymphocytes and macrophages, although fewer numbers are present in chronic rejection

compared with acute rejection.

Macrophages have received much attention in the pathophysiology of lipid-induced atherosclerosis but their role in graft arteriosclerosis is virtually unexplored.[67] Histopathological and immunohistochemical studies of organ allografts with or without clinical evidence of dysfunction have documented the presence of substantial numbers of macrophages in such grafts. This finding suggests the presence of phenotypically different macrophage subpopulations with conceivably distinct functions and cytokine profiles.[68-69] The mechanism by which macrophages are believed to stimulate chronic rejection is by producing cytokines and growth factors.[70-72]

The role of adhesion molecules in chronic rejection

The specific adhesion of cells to other cells is a basic requirement for cell migration and recognition and underlies biological processes as inflammation and immunity. The activation of endothelial cells with the induction of adhesion molecules is primarily unrelated to antigen-dependent events. A broad variety of factors is able to promote the expression of adhesion molecules. These factors include ischemia-reperfusion, surgical manipulation and metabolic factors, such as oxygen radicals or lipids.[73-75] Once endothelial cells are activated and express adhesion molecules, leukocytes may invade the tissue, followed by an episode of cellular inflammation with release of growth factors.[76-78]

In the setting of chronic rejection, few studies have been undertaken to investigate the role of adhesion molecules in the process. Heemann et al. investigated the role of adhesion molecules in a model of chronic kidney rejection in the rat. Intercellular adhesion molecule I (ICAM-1) expression was found immediately before the histologically apparent onset of chronic rejection on small vessels, glomeruli and tubules.[79] In this rat model, ICAM-1 expression appeared to precede the infiltration of lymphocytes and monocytes. In *in-vitro* studies, the same group found adherence of these cells preferentially to sites of ICAM-1 upregulation, which also indirectly suggests a role of ICAM-1 in chronic rejection.[80]

Heemann et al. also investigated the relationship between infection, expression of adhesion molecules and the development of chronic rejection. To test the hypothesis that infection enhances graft arteriosclerosis by increasing expression of adhesion molecules on endothelial

cells, infection was simulated in the rat model of chronic kidney rejection using lipopolysaccharide (LPS). LPS injection was able to induce adhesion molecule expression and was correlated with an accelerated pace of chronic rejection.[81] Therefore, this experiment also suggests that adhesion molecules are involved in the mechanism leading to chronic rejection.

Alloantigen-independent factors in chronic rejection

Based upon experimental and clinical data, alloantigen-independent risk factors are demonstrated to have a role in the development of chronic rejection.[82] These include surgical manipulation and factors related to the donor and the graft. Up to now, it is still unclear if ischemia contributes to the development of chronic rejection as well.[83] Some clinical studies reported a positive influence of ischemia on chronic rejection, whereas other studies found no correlation.[84-87] Experiments in animals also show no consistency with regard to the role of ischemia in graft arteriosclerosis.[88-90]

Several mechanisms of injury from ischemia and reperfusion have been suggested. Endothelial cell injury may occur with increased expression of growth factors like PDGF. Increased graft immunogenicity may arise as the result of increased expression of adhesion molecules and MHC antigens.[91-93] Increased graft immunogenicity may subsequently explain the correlation between ischemic time and the number of acute rejection episodes.[94]

Cytomegalovirus (CMV), a member of the herpesvirus family has been associated with the development of chronic rejection in heart transplantation.[95,96] Experimental studies in the rat showed that CMV infection accelerates intimal hyperplasia in aortic grafts.[97,98] The mechanism by which CMV infection is thought to trigger chronic rejection is by inducing the expression of MHC class II antigens and of adhesion molecules.[99,100]

Experimental studies reported that hypercholesterolemia acts synergistically with allogeneic immune rejection in the development of graft arteriosclerosis.[101] Further evidence supporting the influence of hypercholesterolemia is the use of HMG-CoA reductase inhibitors in clinical transplantation. In heart transplantation, pravastatin was able to reduce the degree of coronary arteriosclerosis compared with nontreated patients.[102] Elevated triglyceride and LDL serum levels have been noted in cardiac transplant patients, but most clinical studies failed to find a strong correlation between the lipoprotein levels and graft arteriosclerosis.[103-105]

Under CsA immunosuppression, the prevalence of hypertension is around 75%. Only in renal transplantation however, hypertension is significantly related to a higher rate of chronic graft failure.[106]

Other non-immune risk factors associated with chronic rejection include posttransplant infections, obesity and the use of kidneys from very young, very old, female or black donors.[17,107,108]

Prevention and treatment of chronic rejection

Since its introduction into clinical heart transplantation in 1980, the fungal metabolite cyclosporine (CsA) has become the basal constituent of immunosuppressive regimens.[109] Its effect is related to the inhibition of the production of interleukin-2 (IL-2), through which it reduces the amplification of T helper cells and the development of mature cytotoxic T cells. CsA very effectively reduces early graft loss from acute rejection, but over time, chronic rejection occurs in the majority of grafts.[110] The lack of efficacy of CsA to prevent chronic rejection may be related to its preferential effect on the T-helper-1 subset of CD4+ cells. By little effect on the T-helper-2 cells, CsA would fail to effectively suppress cytokine production that may help B cells to develop into plasma cells.[111] Another possibility is that CsA directly causes damage to the vessel wall with the development of graft arteriosclerosis.[112] Recently, CsA has been found to upregulate serum levels of the fibrogenic growth factor TGF-β.[113] TGF-β stimulates fibroblast growth and differentiation as well as production of extracellular matrix proteins. This mechanism may explain the contributing effect of CsA on chronic rejection.[113] However, besides accelerating the development of chronic rejection, CsA has also been found to suppress graft arteriosclerosis.[42,114,115]

CsA has various side effects. Most important are hypertension, nephrotoxicity, increase of low-density lipoprotein cholesterol levels, increased risk for infection and cancer.[113,116,117] Side effects of CsA, which are important but have little effect on mortality, include hirsutism, gingival hypertrophy and tremor. Monitoring the trough levels of CsA is necessary to achieve a targeted immunosuppressive level and to prevent unwanted side effects.

In the last decade, two new immunosuppressive agents have been approved for use in clinical transplantation. Mycophenolate mofetil is demonstrated to reduce significantly the rate of acute

rejection in renal transplantation.[118-120] Similarly, tacrolimus (FK506) is approved for use in clinical transplantation after it was demonstrated to be very effective in the prevention of acute allograft rejection.[121] Because these drugs substantially reduce the rate of acute rejection episodes, it is hoped that there will be a long term benefit in decreasing the incidence of chronic rejection.

There is limited experience with therapeutic interventions for allografts suffering from chronic rejection. One clinical study reported the benefits of plasma exchange in 6 renal transplant patients.[122] Other studies have suggested that CsA benefits renal transplant patients who develop chronic rejection while on prednisone and azathioprine therapy.[123-125] Recently, many therapeutic approaches to the prevention of chronic rejection have been evaluated in rat allograft models. These studies reported that high doses of CsA, rapamycin or leflunomide were even able to reverse chronic allograft lesions, which gives a new clinically point of view on the prevention or treatment of chronic rejection.[42,126,127]

1.3 CHRONIC REJECTION AND THE AORTA TRANSPLANTATION MODEL

Introduction

In the study of chronic rejection, several rat heart transplantation models using different rat strain combinations are reported.[128-130] In these models proliferation of the intima develops which resembles vascular lesions of human cardiac grafts suffering from chronic rejection. Because of this similarity, rat heart transplantation models are used frequently in the study of chronic rejection. However, there are important disadvantages linked to this model. To prevent graft loss due to acute rejection, allotransplantation in immunological weak strain combinations or under immunosuppression is necessary. Suppression of acute rejection however, delays the development of chronic rejection.[39-42,131] This explains why chronic rejection in rat heart transplantation models first appears several months post-transplantation, which makes these models time-consuming. Aortic allografts however, are not lost due to acute rejection. Despite acute rejection in the vascular wall, the lumen of aortic allografts remains open. Therefore, aortic allografts do not necessarily require immunosuppression. Non-immunosuppressed aortic allografts may develop arteriosclerosis within 3 weeks, which makes the aorta transplantation model time-efficient compared with the heart transplantation model.[42,132] Another advantage of the aortic transplantation model is the possibility to investigate the effects of individual drugs, including immunosuppressive drugs, on graft arteriosclerosis without employing any unwanted prophylactic immunosuppressive treatment. In 1975 Williams et al. described the rat aorta transplantation model, in which rejection and repair of endothelium was studied.[133] More recently, Schmitz-Rixen et al. described the three components of arterial allograft rejection in the rat aorta transplantation model: (1) intimal thickening, including smooth muscle cells and inflammatory cells; (2) necrosis of the media, with loss of medial smooth muscle cells and a time-dependent attack on the extracellular matrix; (3) and inflammatory cell infiltration, mostly in the adventitia.[134] In 1990, Halttunen et al. re-introduced the aorta transplantation model in the rat as an experimental model of chronic rejection and showed that similar pathogenetic events take place as in human heart transplants suffering from chronic rejection.[132,135] Up to now, the rat aorta transplantation model has been used for various kind of studies, which will be discussed next.

Studies on immunosuppression

In 1988, Schmitz-Rixen et al. reported prominent infiltration of aortic allografts by lymphocytes with intimal proliferation 30 days post-transplantation.[134] CsA treatment suppressed cellular infiltration but partially prevented intimal thickening. Mennander et al. investigated in 1991 the effect of CsA on the development of graft arteriosclerosis.[112,136] Low dose CsA treatment was found to induce transplant arteriosclerosis. CsA was suggested to cause endothelial cell damage of the allograft. This damage might be responsible for the accumulation of inflammatory cells in the subendothelial space with secondary "endothelialitis". Finally, endothelin and possibly other growth factors were suggested to be responsible for the mobilization and proliferation of VSMC leading to graft arteriosclerosis. In our study on the effect of immunosuppression however, we observed that effective suppression of acute rejection by CsA or Rapamycin prevents graft arteriosclerosis in aortic allografts.[42] Similarly, Stoltenberg et al. found that CsA inhibits intimal hyperplasia in rat aortic allografts and Gregory et al. reported similar findings on arteriosclerotic formation in femoral artery allografts.[126,137-139] In the last study effective immunosuppression by rapamycin inhibited intimal thickening by 98%. Recent studies in rat heart and kidney models also support that graft arteriosclerosis can be prevented by effective immunosuppressive therapy.[39-41] That CsA can also prevent graft arteriosclerosis as a result of surgical injury was demonstrated by Bernucci et al.[114] In all non-treated isografts, graft arteriosclerosis was present in the perianastomotic tract of the recipient aorta, whereas it was only found in 3 of 9 treated animals.

Of the new immunosuppressive agents, Räisänen-Sokolowski et al. tested the effect of 15-deoxyspergualin on the development of chronic rejection in rat aortic allografts.[140] They reported that this molecule partially inhibits allograft arteriosclerosis, likely by reducing mRNA synthesis of platelet-derived growth factor-BB, insulin-like growth factor 1, epidermal growth factor and TGF-β1 by macrophages.[141] Furthermore, this group tested mycophenolate mofetil.[142] This new immunosuppressive drug inhibited chronic rejection, but did not affect the mRNA expression of several growth factors as did 15-deoxyspergualin.[143]

The effects of the immunosuppressive drug leflunomide on chronic vascular rejection were evaluated by Kwan et al.[144] Leflunomide significantly inhibited adventitial inflammation as

well as intimal hyperplasia.

A complete different approach to influence the immune system in the aortic transplantation model is described by Gaciong et al.[145] They tested whether graft arteriosclerosis could be affected by proteases, using a combination of trypsin and bromelain. A beneficial effect of proteases was found on allograft arteriosclerosis. This combination of proteases has been shown to affect T cell activation by changing the expression of surface molecules.[146-147]

Studies on alloantigen-independent factors

Plissonnier et al. studied aortic grafts in normotensive and spontaneously hypertensive rats.[148] They observed not only intimal proliferation in the allogeneic combinations, but also in isografts of hypertensive rats. This observation may be explained by a changed VSMC proliferative potential in hypertensive rats. In the literature, several studies have reported that VSMC of spontaneously hypertensive rats are significantly hyperresponsive to platelet-derived growth factor in vivo.[149,150] This hyperresponsiveness would be due to abnormally high levels of phospholipase C activity and Na^+/H^+exchanger, which is probably genetically predisposed. Nevertheless, hypertension might also have a direct effect on the proliferation of VSMC. An increase in VSMC proliferative potential has been reported after salt-induced hypertension.[151] That hypertension is of importance in the development of graft arteriosclerosis is also demonstrated in another study of Plissonnier et al.[152,153] In this study the effect of angiotensin converting enzyme inhibition (ACEI) was tested in the aorta transplantation model by treating the animals with perindopril. Perindopril significantly decreased intimal thickness. This effect of ACEI on intimal thickness was proportional to the decrease in blood pressure and therefore this study supports the hypothesis that hypertension might be involved in the development of transplant arteriosclerosis.

Inconsistent results are reported on the effect of ischemia in the aorta transplantation model. Kouwenhoven et al. found that ischemia did not enhance transplant arteriosclerosis, in contrast to the Helsinki group.[83,154] The last group tested the effect of superoxide dismutase on ischemia-induced arteriosclerosis in syngeneic aortic grafts.[154] Superoxide dismutase reduces the amount of free oxygen radicals, which are believed to mediate the induction of intimal proliferation after prolonged ischemia. Although they found that prolonged ischemia enhanced

graft arteriosclerosis, they reported a lack of effect of superoxide dismutase on ischemia induced arteriosclerosis.

Nitric oxide is suggested to have an effect on graft arteriosclerosis. Nitric oxide is generated in the vascular wall by nitric oxide synthetase, which expression is increased in aortic grafts, especially after prolonged ischemia time.[155]

Another alloantigen-independent risk factor tested is hyperlipidemia, because clinically this appeared to be correlated with chronic rejection. Indeed, in the aorta transplantation model, hyperlipidemia was capable to enhance graft arteriosclerosis.[156]

Studies on alloantigen-dependent factors

Mennander et al. observed focal thickening of the intima associated with starch powder granulomas in syngeneic aortic grafts.[157] Immunohistochemical staining of the IL-2-receptor as an indicator of the activation level of the inflammatory lymphocytes, showed only a low level of lymphoid activation in starch powder granulomas as compared with a strong lymphoid activation in the adventitia of aortic allografts. Therefore, they proposed that the level of lymphoid activation is not associated with intimal proliferation. The same study showed that starch powder-induced intimal thickening in syngeneic aortic grafts is not accompanied by medial necrosis. In aortic allografts on the contrary, medial cell destruction and the development of intimal lesions are closely related. Therefore, they suggested that intimal thickening and medial necrosis may be independently regulated.

Mennander et al. also studied in the aorta transplantation model whether chronic rejection might be reversible.[158] Herefore, retransplantations of aortic allografts with intimal lesions were performed to the donor rat strain. Contrary to the expected, after retransplantation, increasing intimal hyperplasia was observed and therefore they concluded that chronic rejection might be irreversible.

Lemström et al. studied the effect of CMV infection on aortic grafts. CMV infection was shown to accelerate adventitial inflammation and to double the intimal proliferation of aortic allografts.[97] Triple drug immunosuppression containing CsA, prednisone and azathioprine did not only reduce graft arteriosclerosis, but also induced early latency of CMV infection.[159]

Chen et al., who detected upregulation of the immunoglobulin J chain mRNA studied in aortic

grafts the role of humoral antibodies in graft arteriosclerosis.[63]. The J chain is needed for the formation of IgM and IgA molecules and suggests the presence of IgM- or IgA-producing plasma cells.

By using the polymerase chain reaction amplification technique, Isik et al. studied JE gene expression during the early development of acute arterial graft rejection.[160] The JE gene codes for a glycoprotein that attracts monocytes. Because glucocorticoids downregulates JE gene expression, Isik et al. hypothesized that the immunosuppressive action of glucocorticoids may be partially explained by its effect on monocyte infiltration.

Drugs tested in the aorta transplantation model

Besides the effects of immunosuppressive agents, Räisänen-Sokolowski et al. tested the Vitamin D analog, MC1288, in the aorta transplantation model. This drug inhibited adventitial inflammation and was able to suppress the development of intimal lesions.[161] Another drug that is also capable to inhibit chronic rejection in aortic allografts is angiopeptin. Angiopeptin is a somatostatin analogue, which inhibits the growth of VSMC in vitro. Because angiopeptin did not reduce the intensity of perivascular inflammation of aortic allografts, the action of angiopeptin was suggested to be due to a direct effect on VSMC proliferation.[162]

Finally, Akyurek et al. tested the effect of low molecular weight heparin derivatives on transplant arteriosclerosis in the rat transplantation model.[163] They found that heparin derivatives only partially inhibited ischemia-induced syngeneic graft arteriosclerosis, whereas no effect could be demonstrated in allogeneic aortic grafts. Immunohistochemistry showed that the inhibition of graft arteriosclerosis in isografts was associated with a reduction in expression of transforming growth factor β1 and platelet-derived growth factor.

1.4 AIM OF THE THESIS

Chronic rejection has become the predominant cause of late cardiac failure. Up to now little is known about its pathogenesis and treatment. Considering this important clinical fact we introduced the aorta transplantation model into our laboratory, which enabled us to study the pathogenesis and therapeutic approaches of graft arteriosclerosis. In this model, we studied the role of different immunological components on intimal hyperplasia. In addition, the effect of immunosuppression and platelet aggregation was assessed. The studies were started at the Laboratory for Experimental Surgery, Erasmus University Rotterdam and were completed in laboratories of the Department of Immunology and Infectious Diseases and Cardiovascular Surgery at the Johns Hopkins School of Medicine, Baltimore USA.

The aim of these studies was:

1 To characterize the immunological components involved in graft arteriosclerosis (Chapter 2).

2 To analyze the correlation between early immunological events after ischemia-reperfusion and graft arteriosclerosis (Chapter 3).

3 To determine the role of cytokines in the regulation of progressive intimal proliferation (Chapter 4)

4 To assess the effect of immunosuppression on the development of graft arteriosclerosis using rapamycin and cyclosporin (Chapter 5).

5 To analyze the effect of platelet aggregation on the development of graft arteriosclerosis using the drug ketanserin (Chapter 6).

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CHAPTER 2

ACCELERATED ARTERIOSCLEROSIS IN AORTIC GRAFTS: A ROLE FOR ACTIVATED COMPLEMENT AND IGM ANTIBODY IN EARLY LESION DEVELOPMENT

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ABSTRACT

Chronic rejection is the predominant cause of late graft failure. The aim of this study was to characterize immunopathologic events in the aorta transplantation model. Orthotopic infra-renal aortic allografts were performed from LEW→BN (n=21) and BN→LEW (n=21). Controls were: LEW→LEW (n=9) and BN→BN (n=9). Animals were sacrificed at 2, 3, 4 and 8 weeks. Aortic graft tissue was processed for quantitative light microscopy and immunohistochemistry. 19% of the LEW→LEW and BN→BN isografts contained small intimal lesions. After the second week post-transplantation, no growth of these intimal lesions was observed. At 2 weeks, in 22% of the LEW→BN and BN→LEW allografts, intimal lesions were seen, increasing to 87% at 8 weeks.

Immunohistochemistry of the isografts showed only faint, focal intimal depositions of both IgM and complement membrane attack complex (C5b-9), whereas allografts showed more intense and diffuse IgM and C5b-9 staining patterns. In allografts only, induced expression of endothelial ICAM-1 and MHC class II was observed at all timepoints, whereas in isografts no expression was found at 8 weeks. In early intimal lesions, these IgM and C5b-9 depositions were found to be infiltrated mainly by macrophages. Progressive intimal lesions at 4-8 weeks were characterized by dense IgM deposition, increasing numbers of MHC class II positive macrophages, CD4+ and CD8+ T lymphocytes and actin-positive smooth muscle cells. In conclusion, IgM deposition and complement activation are associated with early intimal lesion development in both allografts and isografts. In allografts over time, both antibody-mediated host responses as well as induced expression of ICAM-1 and MHC class II molecules may amplify local migration of macrophages and T cells, resulting in severe vascular inflammation with progressive graft arteriosclerosis. This sequence of immunopathologic events suggests that inhibition of the complement system may reduce chronic rejection.

INTRODUCTION

HLA matching between donor and recipient and the use of the immunosuppressive agent cyclosporine have led to an approximate 80% one year survival of heart allografts.[1] With prolonged graft survival, chronic rejection has evoked as a late complication of heart

transplantation.[2] The key characteristic of chronic heart rejection is concentric intimal thickening over the entire length of the coronary vessels, which gradually reduces the peripheral blood flow. Occlusion of coronary arteries may occur resulting in myocardial infarction, ventricular arrhythmias, or most dramatically, sudden death.[3,4] The development of intimal lesions occurs more rapidly in allogeneic grafts as compared with isografts, which may develop such lesions to a lesser extend.[3,5] This finding provides evidence that allogeneic immune processes play a role in chronic rejection. In this process however, the precise role of humoral and cellular rejection has still not become clear.[6,7]

Chronic rejection has long been thought to be an antibody-mediated event, as immunoglobulin and complement complexes are associated with areas of intimal hyperplasia.[8-10] However, the literature is unclear on this subject as antibodies have been difficult to demonstrate consistently. Cellular rejection may be involved in chronic rejection as well. Studies that focussed on the cellular component of transplant arteriosclerosis found primarily macrophages and T cells in intimal lesions.[10-13] An important role for this cellular infiltrate in the development of chronic rejection is suggested by the correlation between chronic and acute rejection, which is also characterized by interstitial inflammatory infiltrates of T lymphocytes and macrophages.[14-16] In addition, effective suppression of cellular (acute) rejection showed to prevent graft arteriosclerosis.[17] Therefore, it has been proposed that chronic rejection may be the result of subclinically smoldering cellular (acute) rejection due to ineffective immunosuppression.[17-18]

The studies above show that there is still no generally accepted theory on the pathogenesis of chronic rejection. Both the temporal sequence of humoral and cellular immune responses as well as their respective contribution to the development of transplant arteriosclerosis have not yet been defined. Therefore, in the present study we aimed to characterize the immunological components involved in graft arteriosclerosis. We used the rat aorta transplantation model, which shows pathologic features of intimal hyperplasia similar to those found after human cardiac transplantation.[17,19,20]. Our observations constitute the basis of this report.

MATERIALS AND METHODS.

Experimental animals

Inbred Lewis (LEW, $RT1^l$) and Brown Norway (BN, $RT1^n$) rats were used. All animals were obtained from Harlan (Indianapolis, IN) and had free access to food and water. Male rats, weighing 200-250 g and aged 10-12 weeks, were used as recipient and donor. This study conformed to the guidelines specified in the National Institute of Health Guide for the Care and Use of Laboratory Animals and was approved by The Johns Hopkins Animal Care and Use Committee.

Aortic transplantation

Donor and recipient animals were anesthetized with intraperitoneal sodium pentobarbital (60 mg/kg). A segment of the infra-renal aorta, approximately 1 cm long was isolated, excised, perfused with saline and used for transplantation. The recipients were anesthetized with sodium pentobarbital, a similar segment of aorta was removed and the segment of the donor aorta was transplanted into an orthotopic position below the renal artery and above the iliac bifurcation (Fig. 1). End to end anastomosis was performed using a 9.0 monofilament nylon suture (Ethicon, Sommerville, NY). Operating time was less than 60 minutes.

Aortic allografts of the BN→LEW combination as well as the LEW→LEW and BN→BN isografts were harvested at 2, 4 and 8 weeks representing early, mid and late timepoints post-transplantation. Because pilot studies showed that LEW→BN aortic allografts developed graft arteriosclerosis in a shorter time frame than BN→LEW aortic allografts, LEW→BN aortic allografts were harvested at 2, 3 and 4 weeks similarly representing early, mid and late timepoints post-transplantation. The allogeneic groups counted seven animals and the syngeneic groups three animals at each timepoint.

Tissues were processed for routine histology and immunohistochemical staining. Specimens of aortic tissue in addition to segments of the recipient heart and spleen were immersed in OCT compound and stored at -70°C for immunochemical analyses.

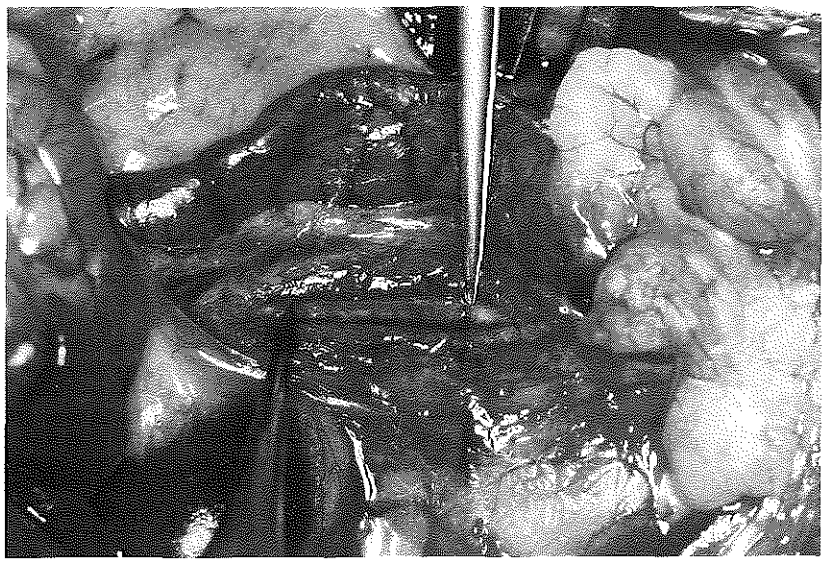

Figure 1. Photograph of donor aorta transplanted into the orthotopic position below the renal artery and above the iliac bifurcation. The proximal and distal anastomoses are noted by the placement of the forceps.

Light microscopy

Snap-frozen tissue sections were stained with hematoxylin and eosin for morphology and with Verhoeff's van Gieson stain for elastic fibers. An automated Zeiss video microscopy system was used to assess mean intimal areas. Quantitative data are expressed as means ± S.D.

Primary antibodies used for immunohistochemistry

Rat anti-mouse MCA 46 (MHC Class II), MCA 341 (Macrophage), MCA 278 (IgG2a), MCA 189 (IgM), MCA 48 (CD8), MCA 55 (CD4), MCA 43 (CLA), MCA 773 (ICAM-1) and mouse anti-rat CD18 (all purchased from Serotec, Harlan Bioproducts for Science, Indianapolis, IN). 2A1 (mouse anti-rat C5b-9) was kindly provided by Dr. William Couser (U of Washington, Seatle, WA). Monoclonal mouse anti-muscle actin was purchased from Enzo Diagnostics, Farmingdale, NY.

Immunohistochemical procedure on rat aortic transplants

Cross-sections of 5μ were prepared of all aortic transplants. Incubations for the immunocytochemical procedure were performed at room temperature in a humidity chamber and all rinses were for 5 minutes each. Sections of non-transplanted WAG and BN aortas were used as controls.

Snap-frozen tissue sections were stored at 4°C in a desiccator over night. They were then fixed in alumina filtered acetone for 10 minutes and rinsed twice in tris buffered saline (TBS). The slides were incubated with 0.1 mg/ml Avidin (Sigma) for 20 minutes to block any natural biotin in the tissue, then rinsed in TBS and incubated with biotin (0.05 mg/ml, Sigma) for 20 minutes. Next, the slides were rinsed in TBS and incubated with 0.05% H_2O_2 in a solution of TBS, 1% normal goat serum (NGS) and 0.5% dried carnation milk for 30 minutes. The slides were then rinsed in a TBS/milk solution (0.5% carnation milk) and blocked with TBS/milk/1%NGS for 10 minutes. After rinsing in TBS/milk, the tissue sections were incubated for 60 minutes with the appropriate primary antibody diluted in a solution of TBS/milk/1%NGS. After incubation, the slides were rinsed in TBS/milk and incubated for 30 minutes with the appropriate biotinylated secondary antibody diluted in TBS/milk/1%NGS. Secondary antibodies utilized included Goat F(ab)'2 anti-Mouse FC IgG, Goat F(ab)'2 anti-Mouse IgM (Jackson Immunoresearch Laboratories, West Grove, PA) and Goat anti-Rabbit IgG (Vector Laboratories, Inc., Burlingame, CA). After incubation, the slides were rinsed in TBS/milk and incubated for 60 minutes with the tertiary Extravidin Peroxidase (Sigma), diluted in TBS. Then they rinsed in TBS/milk, followed by TBS alone. A solution of the chromogen, 3,3 Diaminobenzidine Tetrahydrochloride (Sigma) of 6 mg/ml in TBS with 10µl of 30% H_2O_2) was placed on the slides for 8 min. The slides were then rinsed in water, dipped in $CuSo_4$ (5% copper sulphate in 0.15M NaCL) for two minutes, and stained with mayers modified hematoxylin (Poly Scientific, Bayshore, NY) until the desired intensity was reached. The slides were immersed in tap water for 5 minutes, dehydrated in increasing concentrations of alcohol, cleared with xylene and a coverslip was placed using Permount (Fisher Scientific, Pittsburgh, PA).

Statistics

Comparisons of intimal areas at different timepoints in the two allograft groups versus control

isografts were made using an ANOVA analysis followed by a Fishers PLSD. A p value of 0.05 was considered statistically significant. All statistical analyses were performed using Statview 4.01 (Abacus Concepts, Inc, Berkeley, CA).

RESULTS

Routine histology of aortic transplants

From two weeks on, only 3 of 18 LEW→LEW and BN→BN aortic isografts contained small intimal lesions, which did not increase in size over time. At two weeks, intimal lesions were found in 2 of 9 (22%) of the LEW→BN and BN→LEW aortic allografts, increasing to 20/23 (87%) at the latest timepoints. The adventitial layer in aortic allografts showed extensive inflammation and fibrosis. Medial smooth muscle injury was also noted and was characterized by focal disruption of elastic fibers, vacuolization of smooth muscle cell cytoplasm and a gradual loss of vascular smooth muscle cell nuclei, particularly in areas of intense mononuclear cell infiltration (data not shown).

Quantitative microscopy

The progression in time of mean intimal areas in isografts and allografts is depicted in Fig. 2. In LEW→BN allografts, proliferative intimal lesions were more prominent and developed quicker. In BN→LEW allografts, there also was a progressive, yet relatively milder, increase in the intimal area. LEW→BN allografts at 4 weeks had developed significant more arteriosclerosis than BN→LEW allografts 4 and 8 weeks after transplantation ($p<0.05$).

Immunohistochemistry (Fig. 3 and 4)

From 2 weeks on, immunohistochemistry showed intimal depositions of both IgM and complement membrane attack complex (C5b-9) in both isografts and allografts. However, there were clear differences in the localization and intensity of IgM and complement membrane attack complex (C5b-9) staining in isografts and allografts. In only 3 of 18 isografts, small intimal lesions with focal intimal staining of IgM and C5b-9 were noted. No deposits of IgM and C5b-9 were seen in the medial and adventitial layers. In contrast, in all allografts with intimal hyperplasia, dense IgM and C5b-9 staining was seen in the intimal and

medial layers. No IgM or C5b-9 staining was found in the adventitia. IgM staining persisted in allografts with organized intimal lesions at the latest sacrifice timepoints, whereas the intensity of C5b-9 staining diminished markedly.

IgG accumulation was also present in allografts. In contrast to the IgM deposition, IgG staining clearly increased over time in allografts, was distributed more diffusely throughout all aortic layers and did not specifically localize to sites of intimal proliferation. The switch in the appearance of IgM to IgG antibodies was observed only in allografts, because no IgG staining was found in isografts at any timepoint.

Increased expression of MHC class II and ICAM-1 was initially demonstrated 2 weeks after transplantation, mostly on the endothelial layer, but also in the adventitia. In allografts with advanced intimal lesions, numerous MHC class II positive cells were seen (which were shown to be macrophages by double immunolabeling) in both the intimal and the underlying smooth muscle layers. CD1+ macrophages were seen in the intimal layer and were most prominent in areas of intense IgM and C5b-9 staining. In addition, macrophages were accumulated in the adventitial layer directly opposed to the media. Advanced intimal lesions in allografts at 4 to 8 weeks post-transplantation were infiltrated also by numerous CD4+ and CD8+ T cells and contained actin-positive smooth muscle cells. Throughout the study, no adherence of CD18+ neutrophils to the intimal endothelium was seen.

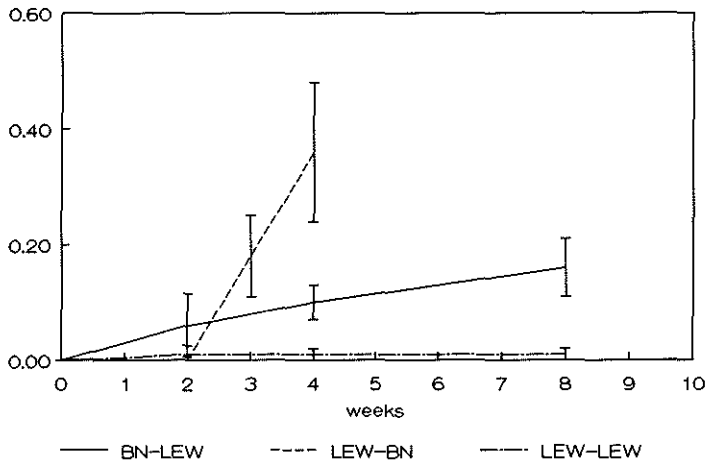

Figure 2. Intimal area in mm^2 at different timepoints posttransplantation in BN→LEW and LEW→BN allografts and LEW and BN isografts.

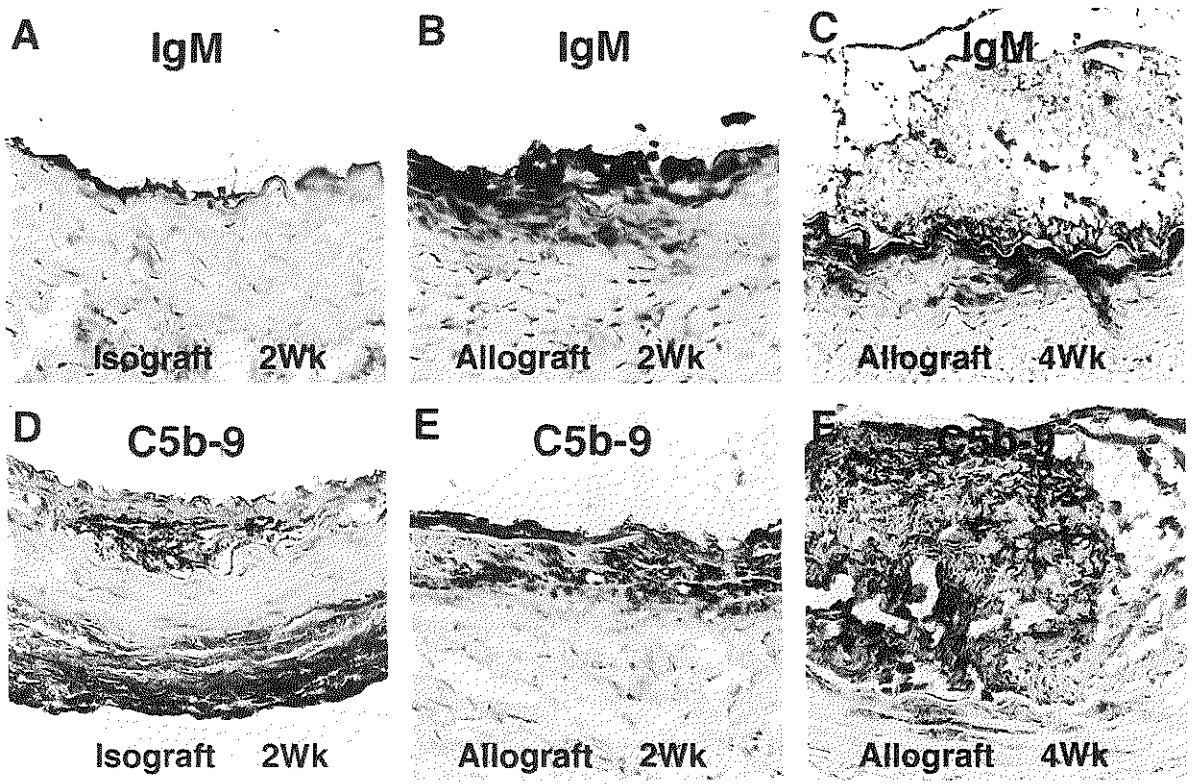
A
IgM
Isograft 2Wk
B
IgM
Allograft 2Wk
C
IgM
Allograft 4Wk
D
C5b-9
Isograft 2Wk
E
C5b-9
Allograft 2Wk
F
C5b-9
Allograft 4Wk

Figure 3. Immunoperoxidase staining of frozen sections taken from representative aortic isografts and allografts sacrificed at 2-4 weeks post-aortic transplantation. (A-C) depicts localization of immunoglobulin (Ig) M antibody by the immunoperoxidase technique. (A) Staining for IgM antibody is limited to the intimal endothelium in a 2 week old aortic isograft (LEW→LEW). (B) More intense staining for IgM antibody in a LEW→BN allograft sacrificed 2 weeks after aortic transplantation. The staining pattern is not limited to the intima. Dense staining of the underlying medial smooth muscle layer is seen. (C) At 4 weeks post-aortic transplantation in a LEW→BN allograft, the intimal proliferative lesion contains scattered positive staining cells as well as dense staining for IgM immediately above the internal elastica. Similar to the staining pattern seen in 2 weeks old allografts, there is dense, widespread staining of the medial smooth muscle layer. (D-F) highlights the localization of C5b-9 by the immunoperoxidase technique. (D) A 2 weeks old BN→BN aortic isograft with a focal intimal lesion which contains a localized, densely staining focus of C5b-9 which is limited to the zone above the internal elastica. This positive staining pattern for C5b-9 was not typical of the majority of isografts sacrificed after 2 weeks post-transplantation, which showed no C5b-9 deposition. The adventitial staining pattern was non-specific and was due to staining by the secundary antibody. (E) A 2 week BN→LEW aortic allograft with dense staining for C5b-9 both on the intimal surface as well as throughout the medial smooth muscle layer. (F) A 4 week BN→LEW aortic allograft with a large, organized intimal lesion with dense staining for C5b-9. The underlying medial smooth muscle layer also stains for C5b-9.

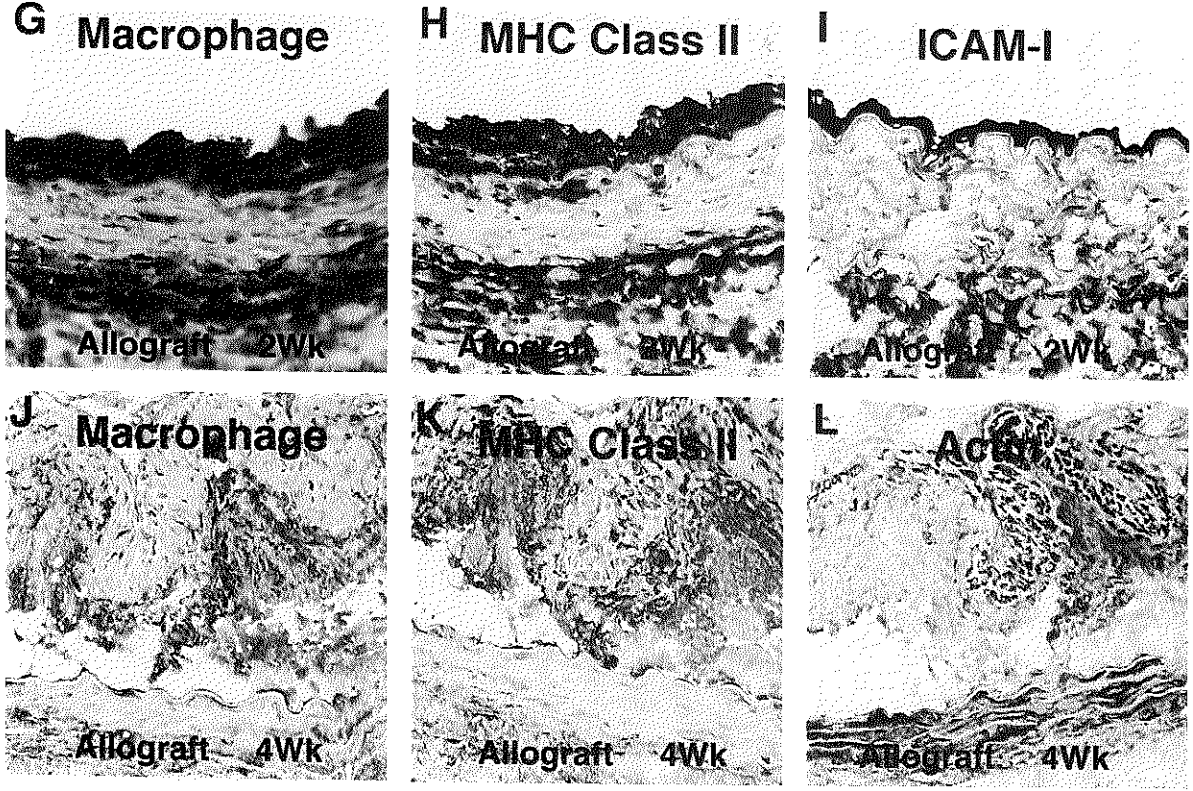
G
Macrophage
Allograft 2Wk
H
MHC Class II
Allograft 2Wk
I
ICAM-I
Allograft 2Wk
J
Macrophage
Allograft 4Wk
K
MHC Class II
Allograft 4Wk
L
Actin
Allograft 4Wk

Figure 4. (G-I) represent immunoperoxidase staining of serial sections taken from a single 2 week old LEW→BN aortic allograft. (G) The intimal lesion shows densely positive staining with the macrophage marker. Macrophages similarly occupy the adventitia immediately adjacent to the medial smooth muscle layer and individual positive-staining cells representing migrating macrophages can be seen within the media. (H) A similar staining pattern is seen for MHC Class II. The intimal lesion as well as the adventitia are strongly MHC Class II positive, while focal positive staining for MHC Class II is noted within the medial smooth muscle. Double immunostaining revealed these migrating MHC Class II positive cells within the media to be primarily macrophages (not shown). (I) Intimal lesions typically stained strongly positive with the ICAM-1 marker. In the area immediately adjacent to that shown in (G & H), the intima shows dense staining for ICAM-1. There is mild, focal staining within the subintimal space and positive staining throughout the adventitia. (J-L) represent immunoperoxidase staining of serial sections taken from a single 4 week old LEW→BN aortic allograft. (J) A well developed intimal lesion with numerous macrophages which appear concentrated within the lower third of the intimal lesion, in the area immediately above the internal elastica. (K) A similar pattern for staining with the MHC Class II marker although, in addition to the positive staining cells above the internal elastica, clusters of cells in the upper third of the intimal lesion are also MHC Class II positive. The majority of these cells in the upper third of the intimal lesion are actin positive and likely represent smooth muscle or smooth muscle-like cells (L). As expected the medial smooth muscle cells stain appropriately positive with the actin marker. (Magnfication A-L, 400X).

DISCUSSION

The present study demonstrates early IgM and C5b-9 depositions in both rat aortic isografts and allografts. These areas contained macrophages and were associated with early intimal proliferation. Antibody-mediated complement activation is therefore likely be involved in early injury leading to graft arteriosclerosis.

Historically, humoral immunity has been implicated as the dominant immunological mechanism that can cause chronic vascular rejection.[21-24] In human heart transplantation, a correlation between the development of chronic rejection and anti-HLA donor specific antibodies has been reported.[25] Also in renal transplantation, immune responses have been shown to be involved in the development of chronic rejection.[7,26] In addition, clinical studies on graft arteriosclerosis showed an association between humoral activity and complement activation.[27,28] This finding suggests that complement activation may, besides humoral immunity, play a role in vascular rejection.

Studies have reported non-allospecific IgM antibodies in the vessel wall of both rat cardiac isografts and allografts as a result of enhanced endothelial permeability.[10,29] In the present study, increased vascular permeability as a response to non-alloimmune injury may also explain the IgM depositions in the aortic isografts. In allografts however, IgM staining was more intense, which suggests that in allografts, besides non-specific antibodies, allospecific antibodies may play a role in the induction of vascular injury.

Only in aortic allografts IgG antibody deposits were observed. Whereas IgM antibodies were associated with early intimal lesions, such a relationship was not found for IgG. IgG deposition clearly increased over time in allografts, was more diffuse than IgM, and did not specifically localize to sites of intimal proliferation. The switch in the appearance from IgM to IgG antibodies in aortic allografts likely represents an allospecific humoral immune response to donor polymorphic antigens. Our study results though, do not point to a clear role for allospecific IgG antibodies in the development of intimal lesions post-aortic transplantation.

In support of our present findings, which demonstrate C5b-9 accumulation in intimal lesions, C5b-9 deposition has also been described in "normally occurring" human atherosclerotic lesions. Similar to what we found in our current study, the extent of C5b-9 present in human

aortic atherosclerosis appears to correlate with lesion size.[30,31] In addition, in experimental atherosclerosis in hypercholesterolemic rabbits, C5b-9 deposition was found to precede monocyte infiltration and foam cell development.[32] Because the present study also shows a correlation between complement deposition, macrophage infiltration and early lesion development, the functional significance of complement in graft arteriosclerosis may therefore depend to a large degree upon its effect on macrophages. C5b-9 complexes and activation-generated complement cleavage fragments can promote the binding and infiltration of macrophages to the vessel wall.[33,34] They have also been shown to stimulate several macrophage functions,[33-36] particularly the release of IL-1 which may increase vascular smooth muscle cell (VSMC) proliferation in vitro,[37] promote endothelial cell adhesiveness for leukocytes, and upregulate the adhesive protein ICAM-1.[37-39] Furthermore, complement may promote graft arteriosclerosis by an effect on the endothelial cell lining. Insertion of C5b-9 into the cell membrane of endothelial cells results in the release of growth factors such as platelet-derived growth factor and fibroblast growth factor. These growth factors in turn can induce VSMC proliferation.[40] In addition, insertion of the complement membrane attack complex into the endothelium can cause endothelial cell activation with increased deposition of platelets on endothelial cells.[41,42] The subsequent release of platelet products would stimulate VSMC to proliferate and migrate into the intima.

In the present study induced expression of endothelial ICAM-1 and MHC class II was observed in both aortic isografts and allografts. Normally, ICAM-1 is constitutively expressed at low levels on endothelial cells. ICAM-1 induction is considered to play an important role in the modulation and maintenance of inflammatory responses by promoting leucocyte adhesion.[43] In humans, endomyocardial biopsies in the immediate post-cardiac transplant period exhibit transient and marked increases in myocardial ICAM-1 expression, which is likely secondary to post-implantation reperfusion injury. Persistence of myocardial ICAM-1 expression though, has been shown to predict the development of future acute rejection episodes, but its role in chronic rejection is as yet poorly understood.[44]

MHC class II expression may also play a role in activating mononuclear cells thus promoting cellular infiltration.[12,45] Increased expression of both ICAM-1 and MHC class II in the current study may thus not only amplify a local vascular inflammation as response to mechanical injury, but also the ongoing allogeneic immune response. In isografts temporal vascular

inflammation may result in the development of self-limiting intimal hyperplasia, whereas persistent vascular inflammation in allografts may result in progressive graft arteriosclerosis.

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CHAPTER 3

IMMUNOPATHOLOGICAL EVENTS EARLY AFTER AORTA TRANSPLANTATION: A STUDY ON ISCHEMIC INJURY

RA Geerling, AA Ansari, AM LaFond-Walker, WA Baumgartner, A Herskowitz.

ABSTRACT

The development of transplant arteriosclerosis has emerged as a major problem to long-term survival after heart transplantation. The accelerated development of arteriosclerosis in transplanted arteries could result either from ischemic injury in connection with surgical trauma, from an immunological reaction against the transplant, or from both. We studied in the rat aorta transplantation model the early immunological events after ischemia-reperfusion for a correlation with graft arteriosclerosis. Orthotopic infra-renal aortic LEW and BN isografts (n=12) and LEW→BN and BN→LEW allografts (n=12) were performed. Non-transplanted aortas were used as controls. The time of exposure to hypoxia of the aortas was 1 hour at room temperature. Animals were sacrificed 3 hours after ischemia-reperfusion and tissues were processed for immunohistochemistry.

After ischemia-reperfusion there was significant denudation of the endothelial cell lining in both isografts and allografts, whereas no damage was observed in non-transplanted controls. In both isografts and allografts, immunohistochemistry showed focal depositions of IgM and complement membrane attack complex (C5b-9) in the intimal and medial layers. The pattern of IgM and C5b-9 staining resembled the lesions found at 2 weeks after aorta transplantation in the same model. No significant deposition of IgG antibody or induction of ICAM-1 and MHC class II expression in either isografts or allografts was seen. Adherence of CD18+ neutrophils to the endothelium was only rarely seen. In conclusion; ischemic injury in the aorta transplantation model is characterized by endothelial cell damage and focal deposits of IgM and C5b-9. In both isografts and allografts, the pattern of these IgM and C5b-9 deposits resembled the immunological findings in aortas harvested at 2 weeks post-transplantation. This suggests that ischemic injury may stimulate the development of graft arteriosclerosis by activation of the complement cascade. In isografts this may result in self-limiting arteriosclerosis, whereas in allografts, subsequent allospecific immune activation may result in progressive graft arteriosclerosis.

INTRODUCTION

Progress in immunosuppressive therapy has improved the prevention and management of acute

rejection in cardiac transplantation. Consequently, the development of a distinct form of coronary arteriosclerosis in the arteries of heart transplants, also called chronic rejection or graft arteriosclerosis, has emerged as a major problem to long-term graft survival.[1,2] Graft arteriosclerosis is a process limited exclusively to the arteries of the allograft and is characterized by progressive intimal hyperplasia.[3,4] This occlusive form of coronary artery disease reduces cardiac perfusion and may result in graft failure.[5] Nowadays, graft arteriosclerosis is the leading cause of death of long-surviving recipients of cardiac allografts.[6,7]

Experimental and clinical studies have proven alloantigen-dependent and -independent factors to be involved in the development of chronic rejection.[8-15] However, it is still unclear if ischemia contributes to this process as well.[16] Some clinical studies showed a positive influence of ischemia on chronic rejection, whereas other studies found no correlation.[17-20] Experiments in animals also show little consistency with regard to the role of ischemia in graft arteriosclerosis.[21,22] In the aorta transplantation model enhancement of graft arteriosclerosis was found after ischemia.[23,24] In contrast, using the same model, in our laboratory no support was found that ischemia may have any impact on graft arteriosclerosis.[16]

In the aorta transplantation model, we recently found a role for complement and IgM antibody in the development of early intimal lesions (chapter 2). The aim of this study was to investigate the immunological events occurring after ischemia-reperfusion and to look for a possible association between ischemia and the development of transplant arteriosclerosis.

MATERIALS AND METHODS

Aortic transplantation

Inbred Lewis (LEW, $RT1^l$) and Brown Norway (BN, $RT1^n$) rats were used as donors and recipients. Aortic transplantation was performed as described in chapter 2.

Graft preservation

Under intraperitoneal sodium pentobarbital (60 mg/kg) anesthesia, a donor segment of infra-renal aorta, approximately 1 cm long, was isolated, excised and perfused with tris buffered saline (TBS). To induce ischemia, the aortas were placed in TBS for 1 hour at room

temperature.

Experimental groups

LEW and BN syngeneic and LEW→BN and BN→LEW allogeneic transplantations were performed. The groups contained 6 animals each. The animals were sacrificed 3 hours after reperfusion and aortas were processed for routine histology and immunohistochemical staining.

Control aortas

Sections prepared from non-transplanted LEW en BN aortas (n=3 in each group), surgically removed and placed in TBS for one hour at room temperature, served as controls. They were similarly processed for histological analysis and immunohistochemistry.

Immunohistochemistry and histology

Immunohistochemical procedures as well as routine histology were performed as described in chapter 2.

RESULTS

Routine histology

Histological examination of the aortic isografts and allografts showed significant endothelial denudation. In allografts however, mural thrombi had developed in 4 out of the 12 rats. The control aortas did not show any abnormalities. There was no evidence of intimal hyperplasia.

Immunohistochemistry (Figure 1)

The non-transplanted controls showed no positive staining for any of the antibodies. Both isografts and allografts showed focal depositions of IgM and C5b-9 in the subendothelial and superficial medial layer. The intensity of the IgM and C5b-9 staining however, was denser in allografts. The most intense staining regions were at sites of mural thrombi. No significant deposition of IgG antibody or induction of ICAM-1 and MHC class II expression was noted in either isografts or allografts. Adherence of CD18+ neutrophils to the intimal endothelium was only rarely seen.

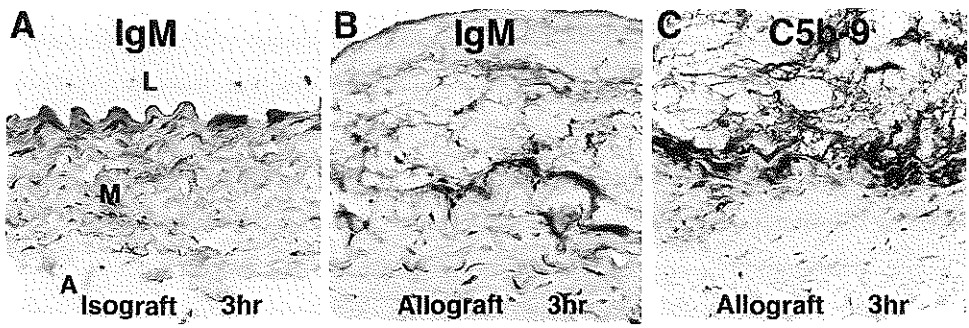

Figure 1. Immunoperoxidase staining of frozen sections taken from representative aortic isografts and allografts sacrificed at 3 hours post-aortic transplantation. (A) LEW→LEW aortic isograft with no demonstrable staining for IgM. A similar lack of significant staining for C5b-9 was also noted in isografts (not shown). (B) LEW→BN aortic allograft with intense staining for IgM on the intimal endothelial surface, which contains an overlying mural thrombus. (C) LEW→BN aortic allograft with a similar overlying mural thrombus as seen in (B). Note the intense staining for C5b-9 on the intimal endothelial surface as well as in the subintimal space and the mural thrombus. Focal staining deep in the medial smooth muscle layer was also noted. (A-C, Magnification, 400X).

DISCUSSION

The present study shows IgM antibody and complement depositions in the vascular wall after 3 hours of ischemia-reperfusion. In both isografts and allografts, this ischemic injury resembled the immunological findings in aortas with early intimal lesions as described in chapter 2. This suggests that ischemic injury may stimulate the development of graft arteriosclerosis by activation of the complement cascade.

Complement complexes have been demonstrated in vessel walls after ischemic endothelial cell injury followed by reperfusion.[25-31] These complement fragments as C5b-9 may promote the

development of graft arteriosclerosis by various mechanisms, which are described in detail in chapter 2.

Not only complement, but also IgM antibody deposits were found in the vascular wall after ischemia-reperfusion. This may be due to increased vascular permeability as the result of denudation of the endothelial cell lining as observed in the present study. Previously, in rat aortas, increased vascular permeability with necrosis of endothelial cells was demonstrated after 1 hour of ischemia.[32,33] Increased vascular permeability however, does not explain the differences in the intensity of staining of IgM and C5b-9 between isografts and allografts. Therefore, we hypothesize that in the aorta transplantation model ischemia-reperfusion leads to influx of naturally occurring IgM with complement activation, representing "response to injury". In allografts however, in addition to these natural antibodies, other antibodies possibly related to blood type alloantigens or other polymorphic antigens expressed by the grafted tissue may play a role in exacerbating intimal injury.

In the present study, no significantly increased expression of endothelial MHC class II or ICAM-1 was found. This may be explained by the short period of reperfusion, because at 2 weeks after transplantation, we found increased expression of MHC class II and ICAM-1 in both isografts and allografts (chapter 2). The upregulation of MHC antigens may lead in allografts to progressive graft arteriosclerosis by promoting humoral and cellular rejection.[34,35] ICAM-1 expression is reported to predict the development of acute rejection episodes in cardiac transplantation, but its role in chronic cardiac rejection is yet poorly understood.[36]

A causal role for ischemic injury in the development of graft rejection is demonstrated by Land et al.[37] This group found that superoxide dismutase reduced both acute rejection episodes as well as chronic rejection in kidney transplant recipients. Superoxide dismutase is thought to reduce graft rejection by reducing the amount of free oxygen radicals, which are believed to mediate the induction of intimal proliferation after prolonged ischemia.

Thus, in the present study we found support for antibody-mediated complement activation in ischemia reperfusion, which may be associated with the development of graft arteriosclerosis. This observation suggests that if ischemic injury stimulates graft arteriosclerosis, chronic rejection may be reduced by inhibition of the complement system.

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CHAPTER 4

ACCELERATED ARTERIOSCLEROSIS IN AORTIC GRAFTS: A ROLE FOR CYTOKINES IN PROGRESSIVE INTIMAL LESION DEVELOPMENT

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This chapter is a modified version of the article:
Accelerated arteriosclerosis in aortic grafts: a role for cytokines in progressive intimal lesion development.
Transplantation Proceedings 1998;30:946-947.

ABSTRACT

Rat aortic allografts develop accelerated arteriosclerosis, which is histologically similar to that observed in chronically rejecting vascularized allografts. We have recently investigated the role of humoral and cell-mediated graft rejection in aortic grafts. In the present study we investigated whether cytokines play a role in the regulation of progressive intimal proliferation. Aortic transplants were performed in the high responder LEW→BN rat combination with progressive arteriosclerosis and in the low responder BN→LEW combination with self-limiting arteriosclerosis. LEW→LEW aortic transplants were done as controls. Grafts were harvested at 2, 3, 4 and 8 weeks. Rat-specific oligonucleotide probes were used to assess the expression of mRNA for TNF-α, IL-1β, IL-2, IL-6, IFN-γ and TGF-β1 with the reverse transcriptase polymerase chain reaction amplification technique. Gene expression for cytokines IL-6, IL-1β, IL-2 and IFN-γ was noted only in allografts, whereas mRNA gene expression for TGF-β1 and TNF-α was noted in both allografts and isografts. Cytokine gene expression was noted only at 2, 3 and 4 weeks in BN→LEW allografts (self-limiting arteriosclerosis), whereas mRNA levels were found at all timepoints in LEW→BN allografts (progressive arteriosclerosis). Therefore, there was a relationship between cytokine gene expression and progression of intimal lesions.

These results provide evidence for local expression of cytokine mRNA in aortic allografts and suggest that cytokines play a role in the process of graft arteriosclerosis.

INTRODUCTION

Accelerated graft arteriosclerosis (chronic rejection) is the predominant cause of graft failure in long-term survivors of cardiac allografts. Graft arteriosclerosis consists of a concentric proliferative intima that is distributed diffusely in the coronary arteries and gradually reduces the peripheral blood flow. When severe, this may result in myocardial infarction, ventricular arrhythmias or sudden death. Although the precise pathogenic mechanisms involved in the development of graft arteriosclerosis remain uncertain, many investigators consider it a form of chronic vascular rejection.[1,2]

In acute graft rejection, an important role has been ascribed to cytokines.[3-6] Cytokines are

thought to be of importance in rejection of allografts via the "cytokine adhesion molecule cascade". When endothelial cells are activated by surgical manipulation, ischemia, or by circulating host antibody, they start to produce cytokines, which in turn induce expression of adhesion molecules. Leucocytes invade the tissue and, depending on the responsiveness of the host, a more or less pronounced episode of acute rejection occurs.[7-13] In chronic rejection however, little is known about the role of cytokines. Some investigators have suggested that cytokines are likely involved in chronic rejection, but from a technical point of view it was not possible to show a direct relationship between cytokine expression and the development of graft arteriosclerosis.[14-22]

We have recently investigated the effect of immunosuppression on posttransplant graft arteriosclerosis in the rat aorta transplantation model.[23] In this model we found a relationship between acute rejection and the development of graft arteriosclerosis. In addition, we observed that effective suppression of acute rejection was able to prevent the development of intimal lesions. In the present study we investigated in the aorta transplant model whether cytokines are associated with the development of transplant arteriosclerosis.

MATERIALS AND METHODS

Aortic transplantation

Inbred Lewis (LEW, $RT1^l$) and Brown Norway (BN, $RT1^n$) rats were used as donors and recipients. Aortic transplantation was performed as described in chapter 2.

Experimental design

Based on pilot studies, we determined that LEW→BN aortic allografts developed graft arteriosclerosis in a shorter time frame as compared with BN→LEW aortic allografts. Therefore, LEW→BN aortic allografts were harvested at 2, 3 and 4 weeks representing early, mid and late timepoints post-transplantation. Aortic allografts of the BN→LEW combination on the other hand as well as LEW-LEW isografts were harvested at 2, 4 and 8 weeks similarly representing early, mid and late timepoints post-transplantation (Table 1). At sacrifice, tissues were processed for immunohistochemistry and for mRNA isolation and polymerase chain reaction amplification.

Table 1. Killing timepoints posttransplantation.

	early	middle	late
LEW-BN allografts (n=20)	2 weeks	3 weeks	4 weeks
BN-LEW allografts (n=22)	2 weeks	4 weeks	8 weeks
LEW-LEW isografts (n=20)	2 weeks	4 weeks	8 weeks

RNA isolation and polymerase chain reaction amplification

Total cellular RNA was extracted from 2 aortic grafts of the different groups at the 2, 3, 4 and 8 week sacrifice timepoints. Approximately 1 cm of rat aortic graft was homogenized with 800 ul RNAZOL STAT (Teltest, Friendswood, TX) in a 1.5 ml microfuge tube, after which 80 ul chloroform was added. After vigorous vortexing the mixture underwent centrifugation and the aqueous phase transferred to a new microfuge tube containing an equal volume isopropanol and the RNA recovered by precipitation by chilling at -20°C for 1 hour, 2 ug of total RNA was subjected to first-strand cDNA synthesis in a 20 ul reaction containing 50 mmol/L Tris-HCl (Ph 8.3 at 42°C), 20 mmol/L KCl, 10 mmol/L $MgCl_2$, 5 mmol/L dithiothreitol, 1 mmol/L of each dNTP, 20 ug/ml oligo-dT(15) and 20 units AMV reverse transcriptase (Boehringer-Mannheim, Indianapolis, IN) for 40 minutes at 42°C. After completion of first-strand synthesis, the reaction was diluted to 100 ul with distilled water and 5 ul was used for each Polymerase chain reaction (PCR). PCR reactions (in a volume of 50 ul) contained 200 umol/L of each dNTP, 1 umol/L of each specific primer, buffer as supplied with the Taq polymerase (Boehringer-Mannheim) and 2.5 units Taq polymerase (Boehringer-Mannheim). The primers were designed to amplify a product of between 250 and 500 nucleotides in length (Table 2) and were also designed to cross introns to avoid confusion between cytokine mRNA expression and genomic contamination. The PCR reaction was performed at 3 different cycle numbers to ensure it was performing in the linear range at which there is a fixed relationship between input RNA and densitometric readout. The optimal cycle number for the monokines (TNF-α, IL-1β, IL-6 and TGF-β1) was 25 and for the lymphokines (IFN-γ and IL-2) 35. A "9600 thermal cycler" (Cetus Corp., USA.) was used.

Target	3′ Oligonucleotide	5′ Oligonucleotide	Product size (NT)	Probe
GAPDH	CTCAGTGTAGCCCAGGATGC	ACCACCATGGAGAAGGCTGG	508	GTGGAAGGACTCATGACCAGTCCATGCC
IL-1β	CTCTGCTTGAGAGGTGCTGATGTAC	GAAGCTGTGGCAGCTACCTATGTCT	520	CTGGAGAGTGTGGATCCCAAACAATACCCA
IL-2	GAGCCCTTGGGGCTTACAAAAAG	CAGGTGCTCCTGAGAGGGHTCG	500	GCCAATTCGATGATGAGCACGAACTGTGG
IL-6	CTAGGTTTGCCGAGTAGACCTCA-TAGTGACC	ATGAAGTTTCTCTCCGCAAGAGACTTCCAG-CCAG	636	GGTCTGTTGTGGGTGGTATCCTCTGTGAAGTC-TCCTCTCCGGACTTGTG
IFN-γ	TCAGCACCGACTCCTT-TTCCGCTTCCTTAGGC	GTTACTGCCAAGGCACACTCATTGAAAGCC	413	GACAACCAGGCCATCAGCAACAACATAAGT
TGF-β1	GACGTCAAAAGACAGCCACT	GAAGCCATCCGTGGCCAGAT	461	TCTCTGCAAGCGCAGCTCTGCACGGG-ACAGCAA
TNF-α	GGACTCCGTGATGTCTAAGT	CACGCTCTTCTGTCTACTGA	546	TGAGAAGATGATCTGAGTGTGAGGGTCTGG

Table 2. Sequences of the oligonucleotide primers used for PCR amplification for cytokine mRNAs, product sizes predicted, and the sequences of the internal oligonucleotides used for southern blot analysis of amplified products.

PCR negative control was without cDNA (H_2O) and positive PCR control was a known positive cDNA. cDNA negative control contained no RNA (H_2O), whereas the positive cDNA control was a known positive RNA. In addition, the amount of PCR product was determined by comparison of signal density to that of standard curves from simultaneously amplified serial dilutions of a positive control for the cytokine of interest and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Generation of these standard curves ensured a fixed relationship between the initial RNA input and the densitometric read-out.

A portion of the PCR reaction product (25%) was electrophoresed through a 1.2% agarose gel and transferred to nitrocellulose. Filters were prehybridized in 2X SSC containing 0.2% Ficoll, 0.2% polyvinylpyrollidone, 0.2% bovine serum albumin, 2 mmol/L sodium pyrophosphate, 1 mmol/L ATP, and 50 ug/ml *Escherichia coli* tRNA at 55°C for 3 to 4 hours. Hybridization was in the same buffer containing 0.1% SDS at 55°C for 12 to 14 hours. Oligonucleotide probes (Table 1) internal to the PCR primers were radiolabelled with $[^{32}P]$ ATP by T4 polynucleotide kinase. After hybridization, filters were washed in 6X SSC, 0.1% SDS at 55°C and finally in 2X SSC at 55°C before autoradiography. The relative radioactivity for bands on autoradiograms was estimated by volume integration by laser scanning densitometry (Molecular Dynamics, Sunnyvale, CA). The relative intensity of bands for cytokine mRNA was normalized using the intensity of the autoradiogram for the internal control, GAPDH.

RESULTS

Quantitative microscopy

The progression over time of mean intimal areas in isografts and allografts is depicted in Fig. 1. Intimal areas in isografts remained small whereas intimal areas in allografts showed a progressive increase in size. LEW→BN allografts developed greater increase in intimal area after 2 weeks than BN→LEW allografts. LEW→BN allografts at 4 weeks had developed significant more arteriosclerosis than 4 and 8 weeks old BN LEW allografts ($p<0.05$).

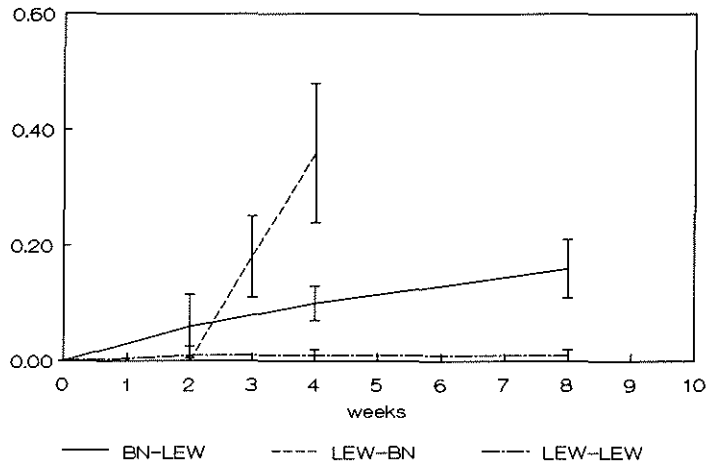

Figure 1. Intimal area in mm^2 at different timepoints posttransplantation in BN→LEW and LEW→BN allografts and LEW and BN isografts.

RNA isolation and polymerase chain reaction amplification

Fig. 2 is composed of representative autoradiograms showing mRNA expression for TNF-α, IL-1β, IL-2, IL-6, TGF-β and IFN-γ with standard dilution curves for each cytokine. Aortic cytokine mRNA expression for TGF-β1 and TNF-α were observed in allografts, isografts and a non-transplanted control. Cytokine mRNA expression for IL-1β, IL-2, IL-6 and IFN-γ was only demonstrated in allografts. In the LEW→BN combination, which develops intimal lesions more rapidly, these mRNA expressions remained high, whereas the expressions in the "weak" BN→LEW combination diminished after 2 weeks post-transplantation.

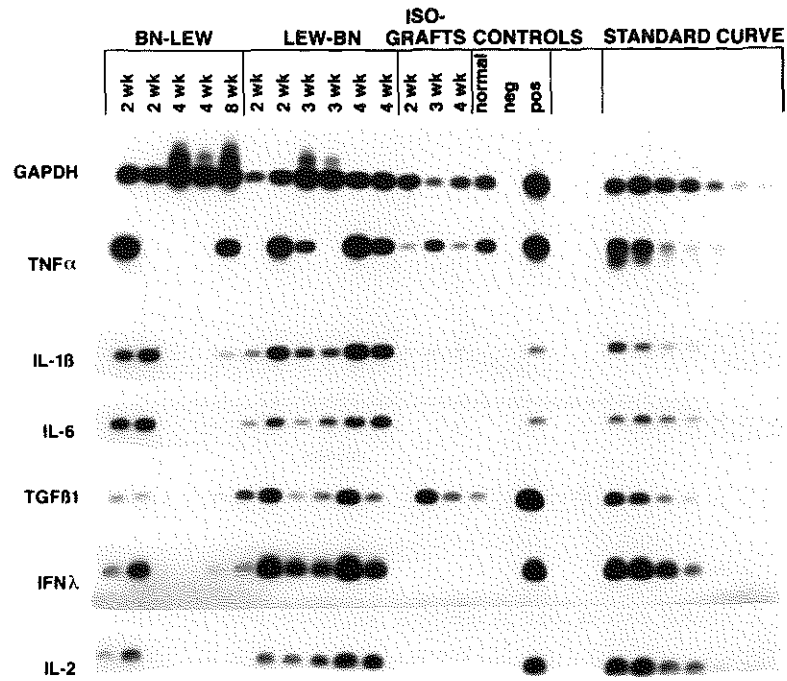

Figure 2. Representative autoradiograms highlighting mRNA expression for the following cytokines: TNF-α, IL-1β, IL-2, IL-6, IFN-γ and TGF-β1

DISCUSSION

This study demonstrates a relationship between vascular mRNA cytokine expression and the severity of graft arteriosclerosis. Cytokine mRNA expression for IL-1β, IL-2, IL-6 and IFN-γ was only demonstrated in allografts. In the LEW→BN combination, which develops intimal lesions more rapidly, mRNA expression remained high, whereas the expression in the "weak" BN→LEW combination diminished 2 weeks after transplantation. These results provide evidence for local expression of cytokine mRNA in graft arteriosclerosis and suggest that cytokines play a role in the process of graft arteriosclerosis.

Cytokines have been associated with chronic rejection.[14-22] Especially IL-1 has been proposed to induce arteriosclerosis. Its major cellular source is the activated monocyte/macrophage and LPS, TNF, and IL-1 itself trigger its production.[24,25] IL-1 acts on macrophages and vascular

endothelial cells by inducing synthesis of IL-6, IL-8 and IL-1. These cytokines are able to upregulate expression of adhesion molecules on endothelial cells and mediate synthesis of prostaglandins.[26-28] Furthermore, IL-1 costimulates T cell activation and antibody production by B cells. These qualities make IL-1 capable to activate local inflammation, but in vitro, IL-1 has also been shown to induce vascular smooth muscle cell proliferation.[29]

Besides IL-1, we also found increased mRNA expression for cytokines IL-2, IL-6 and IFN-γ in aortic allografts. IL-2 has been found to be an autocrine growth factor for T-lymphocytes and to stimulate production of other cytokines by T-cells.[30-32] IFN-γ is secreted by activated CD4+ and CD8+ T cells. IFN-γ is a potent activator of monocytes/macrophages and promotes CD4+ T cell adhesion and extravazation.[33] It also causes vascular endothelium to express MHC class II and acts directly on T and B cells to promote their differentiation and activates NK cells and neutrophils.[33]

IL-6 is of special interest in the development of graft arteriosclerosis. IL-6 is a principal growth factor for activated B cells and induces their differentiation.[34] Because chronic rejection is thought to be partially antibody-mediated, differentiation and activation of B cells by IL-6 may stimulate chronic rejection. This hypothesis is supported by results of previous studies in which we found a role for IgM antibodies and complement in early intimal lesion development.[35]

In both allografts and isografts, mRNA expression for TGF-β1 and TNF-α was noted. TGF-β1 may be a mediator of chronic rejection by upregulating fibroblast growth and differentiation as well as production of extracellular matrix proteins.[36] TNF-α upregulates local inflammation by many different ways. It stimulates endothelial cells, neutrophils and monocytes and induces the expression of MHC class I and II molecules. In addition it is a co-stimulator for T cell-activation and antibody production by B cells.[37,38]

In the present study, in aortic isografts only significantly increased mRNA expression for TNF-α and TGF-β1 was found. Because after syngeneic transplantation no graft-specific immune activation occurs, TNF-α and TGF-β1 likely play a role in the development of arterial intimal thickening as a result of mechanical injury. Cytokine mRNA profiles for IL-1β, IL-6, IFN-γ and IL-2 were also increased during progressive intimal proliferation. These cytokines may therefore be upregulated in the active host immune response to donor specific antigen and are likely essential for the progression of graft arteriosclerosis.

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CHAPTER 5

SUPPRESSION OF ACUTE REJECTION PREVENTS GRAFT ARTERIOSCLEROSIS AFTER ALLOGENEIC AORTA TRANSPLANTATION IN THE RAT

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ABSTRACT

Rat aortic allografts develop arteriosclerotic alterations in the vascular wall that are histologically similar to those observed in chronic rejecting vascular allografts. We used cyclosporine (CsA) and rapamycin (RAPA) in two different rat strain aorta transplantation models to investigate the effect of immunosuppression on posttransplant graft arteriosclerosis. High dose CsA (25mg/kg/3 times/week) treatment significantly inhibited intimal proliferation in the "strong" WAG→BN model ($P<0.01$) as well as in the "weak" BN→WAG combination ($P<0.001$), compared with untreated allogeneic controls. In the latter combination even fewer intimal lesions were present than in WAG autotransplants, suggesting that CsA may also inhibit the arteriosclerotic response to mechanical injury. RAPA (3mg/kg/3 times/week) was as effective as CsA in reducing intimal lesions ($P<0.01$ and $P<0.001$ in the BN→WAG and WAG→BN model, respectively). Low dose CsA (5mg/kg/3 times/week) was only partially effective in preventing intimal lesions.

Histology of the nontreated allografts showed ongoing acute rejection in the adventitial layer. The degree of cellular infiltration correlated with the severity of arteriosclerotic lesions. High dose CsA and RAPA treatment prevented adventitial infiltration in both models, whereas low dose CsA was only moderately effective. In conclusion, in the present study the degree of arteriosclerotic involvement after allogeneic aorta transplantation was related to the severity of cellular adventitial infiltration. The myointimal thickening was inhibited by effective immunosuppressive treatment. These observations may imply that chronic rejection develops after ineffective immunosuppression.

INTRODUCTION

The use of cyclosporine (CsA) in clinical allografting has resulted in prolonged graft survival by the prevention of acute rejection episodes. The improved graft survival to greater than 80% for heart allografts in the first year after transplantation has, however, led to the recognition of a late complication, i.e., the development of accelerated graft arteriosclerosis.[1] This development of graft arteriosclerosis, also called chronic rejection, has been reported to be a major cause of long-term morbidity and mortality after cardiac transplantation.[2,3] Heart graft

arteriosclerosis consists of a concentric thickening of the intima that is distributed diffusely in the coronary arteries and that gradually reduces the peripheral blood flow. Immunocytochemical studies have shown these lesions to be composed of vascular smooth muscle cells and a cellular infiltrate consisting of predominantly macrophages and T cells.[4-6] Arteriosclerosis can result in occlusion of the vessels and congestive heart failure, myocardial infarction, ventricular arrhythmias, and sudden death may represent the endstage of this process.[7] Five years after transplantation, 42-60% of grafts have significant graft arteriosclerosis.[8] There is still no treatment to prevent graft arteriosclerosis and retransplantation remains the only effective therapy.[7]

The pathogenesis of graft arteriosclerosis has not yet been elucidated, but the general believe is that immunological phenomena linked to histocompatibility differences between donor and recipient finally result in the development of proliferative intimal lesions.[9,10] Contradictory reports have been published about the effect of CsA on the incidence of graft arteriosclerosis. Suppression of the host immune response with CsA has been described to reduce the arteriosclerotic changes after allogeneic heart transplantation.[11,12] In earlier studies, we found that CsA could inhibit the development of chronic kidney graft rejection in the BN→WAG rat model.[13] On the other hand, some investigators suggest that CsA may stimulate graft arteriosclerosis by inducing hypercholesterolemia and hypertension.[14,15] Other investigators suggest that CsA may enhance the development of graft arteriosclerosis in vivo more directly by damaging the endothelial lining.[16,17] In vitro studies show direct toxic effects of CsA on vascular endothelial and smooth muscle cells.[18,19] Because of these contradictory reports and poorly defined pathogenetic factors, there is still no generally accepted therapeutic strategy to prevent graft arteriosclerosis.

Several studies have shown that graft arteriosclerosis also develops in the vascular wall after allogeneic transplantation of rat aortas.[20,21] In this transplantation model, we tested whether the degree of arteriosclerotic alterations would be correlated with the severity of rejection. Therefore we used the immunologically "strong" WAG to BN and the "weak" BN to WAG donor-host combinations, as described in earlier studies.[22] Because of the previously found beneficial effects of CsA on long-term BN to WAG kidney graft survival, we examined in these two models the effect of CsA on graft arteriosclerosis. We also investigated the efficacy of rapamycin (RAPA) in preventing graft arteriosclerosis. RAPA was chosen not only for its

immunosuppressive capacity, but also for its reported ability to inhibit smooth muscle cell proliferation after mechanical trauma.[23] Therefore, RAPA might be more effective than CsA in reducing arteriosclerotic lesions.

MATERIALS AND METHODS

Experimental animals

Male inbred Brown Norway (BN, RT1^n) and WAG (RT1^u) rats were used. All animals were obtained from Harlan CPB, Austerlitz, The Netherlands and had free access to food and water. Rats of both strains, 10-12 weeks old, weighing 200-250 g, were used as recipients and donors. The experimental protocols were approved by the Committee on Animal Research of Erasmus University, Rotterdam, the Netherlands, and adhered to the rules established in the *Guidelines on the Protection of Experimental animals* by the Council of the EC (1986).

Immunosuppression

CsA (Sandimmune, Sandoz, Basle, Switzerland) was diluted in olive oil to a final concentration of 50 mg/ml and administered intramuscularly in a low (5mg/kg) and a high dose (25mg/kg) 3 times a week beginning on the day of transplantation.

RAPA (a gift from Wyeth-Ayerst, Princeton, NY) was diluted to a 2.5-mg/ml concentration in Tween 80/carboxymethylcellulose according to the manufacturer's instructions. RAPA was given intraperitoneally in a dose of 3mg/kg 3 times a week beginning on the day of transplantation.

Aorta transplantation

All rats were anesthetized with ether, after which a laparotomy was performed. In both the donor and recipient, a segment of infrarenal aorta, approximately 1 cm long, was isolated, excised, perfused with saline, and used as transplant. Donor aorta was transplanted into the orthotopic position. End-to-end anastomosis was performed by continuous-suturing 9.0 monofilament nylon (Ethicon, Inc., Sommerville, N.J, U.S.A). The ischemic time was between 20 and 30 minutes.

Experimental groups

WAG and BN autotransplantations and BN→WAG and WAG→BN allotransplantations were performed. Untreated WAG→BN aortic allografts were removed at 0.5, 1, 2, 3, 4, 8, 12 and 16 weeks after transplantation. Untreated aortic autografts were removed at 0.5 2, 4, 8 and 16 weeks after transplantation. Two untreated autografts and 4 untreated allogeneic transplants were killed at each timepoint of death. The numbers of animals treated with the different immunosuppressive regimens as well as the numbers of untreated controls killed at corresponding timepoints are listed in Tables 1 and 2. CsA and RAPA-treated allografts were removed in the WAG→BN combination at 4 weeks and in the BN→WAG combination at 8 weeks after transplantation.

Histology

Tissues were embedded in paraffin; straight cross-sections of 5 μm were prepared at 3 levels of the midportion of the graft and stained with hematoxylin eosin and also stained according to elastic of Gieson. Slides were then scored in a blinded fashion by light microscopy. Quantitative histology was done using a calibrated ocular micrometer. The following variables were evaluated: intimal and medial thicknesses, adventitial infiltration and smooth muscle cell necrosis. The average medial thickness and maximal intimal thickness were determinated. Cellularity of the adventitia and media was assessed by counting the number of nuclei at 5 locations. The mean score was multiplied to a field of 0.1 mm^2. Mean $\pm$ S.D. was calculated for all parameters. For statistical analysis of the data, analysis of variance (ANOVA), followed by a student-Newman-Keuls *t* test was performed.

RESULTS

Histology of untreated allografts

For evaluating the progress of vascular alterations after allogeneic transplantation in the present two aortic transplantation models, 60 allotransplants were performed successfully. Concentric graft arteriosclerosis developed in aortic allografts within several weeks after transplantation (Fig. 1). In the WAG→BN model, the process of intimal thickening was very intense, and at 4 weeks, an intimal layer with a thickness of 155 $\pm$ 51 μm had already

developed. All allografts in this strain combination removed at later time points were, due to the intense intimal proliferation, thrombosed and necrotic and therefore not useful for evaluation.

In the reverse combination, from the BN→WAG, intimal lesions developed more slowly. In this rat combination, there was a steady increase of intimal thickness to 171 ± 60 μm at 8 weeks. Intimal thickness reached its maximum at 12 weeks. Then, the process of intimal proliferation stopped, and at 16 weeks, even less graft arteriosclerosis was observed. The adventitial layer showed a fast onset of mononuclear cell inflammation. Fig. 2 shows the changes in cellular infiltration of this layer. In the WAG→BN combination, the ongoing rejection was more severe than in the BN→WAG model. In the latter combination, cellular intensity peaked at 8 weeks, after which subsequently a complete absence of infiltrating cells was observed at 16 weeks. The intensity of cellular adventitial infiltration coincided with the progress of intimal thickening in both models. Fig. 3 and 4 are representative photographs of a 4-week WAG→BN and a BN→WAG aortic allograft, respectively.

Table 1. The effects of immunosuppressive treatment on the intima, media, and adventitia in the BN to WAG model.

Group	Strain combination	n	treatment	week	Intima thickness[a]	Media thickness[a]	Media nuclei[b]	Adventitia nuclei[b]
1	WAG-WAG	3	-	8	51±19	90±3	204±6	104±23
2	BN-WAG	9	-	8	171±60	54±7	36±22	484±188
3	BN-WAG	4	CsA 5mg	8	75±65*	78±16	176±76	341±67
4	BN-WAG	6	CsA 25mg	8	26±13**	82±9	168±34	165±22
5	BN-WAG	5	RAPA 3mg	8	56±23***	85±9	184±27	131±46

Results are presented as means ± S.D. *P<0.05 vs. group 2, **P<0.001 vs. 2 and P=0.05 vs. 1, ***P<0.01 vs. 2.

[a]The intimal and medial thicknesses are expressed in μm.

[b]Numbers of nuclei in the media and adventitia are multiplied to a field of 0.1 mm^2.

Table 2. The effects of immunosuppressive treatment on the intima, media, and adventitia in the WAG to BN model.

Group	Strain combination	n	treatment	week	Intima thickness[a]	Media thickness[a]	Media nuclei[b]	Adventitia nuclei[b]
6	BN-BN	3	-	4	43±11	96±6	228±40	133±9
7	WAG-BN	6	-	4	155±51	69±5	120±40	1014±520
8	WAG-BN	4	CsA 5mg	4	152±118*	94±17	164±49	328±98
9	WAG-BN	5	CsA 25mg	4	52±46**	99±12	168±41	152±40
10	WAG-BN	6	RAPA 3mg	4	32±31***	95±11	171±17	173±68

Results are given as mean ± S.D. *Not significant compared with group 7, **P<0.01 vs. 7, ***P<0.001 vs. 7.

[a]The intimal and medial thicknesses are expressed in μm.

[b]Numbers of nuclei in the media and adventitia are multiplied to a field of 0.1 mm^2.

After transplantation, there was a gradual loss of nuclei in the media (Fig. 5). Eight weeks after transplantation, the media was essentially acellular in the BN→WAG model. Decrease of medial thickness to 50% was observed after 16 weeks (Fig. 6). The elastic staining showed disruption of elastic fibers and interruptions of the internal elastic lamina in the allogeneic groups.

Histology of untreated autografts

Intimal lesions were In 13 of the 22 autografts. These lesions were localized concentrically and were mild (51 ± 19 μm in the BN and 43 ± 11 μm in the WAG) 1 month after surgery. No intimal increase was seen after this period. A similar correlation between cellular infiltration and the progress of intimal proliferation as seen in the aortic allografts was observed in the autografts. The degree of adventitial mononuclear cell infiltration, however, was weaker than in the allografts, peaked at 2 weeks, and returned to normal values thereafter. Medial smooth muscle cell density and medial thickness remained normal throughout the observation period.

Histology of aortic allografts after immunosuppression

For testing the effect of CsA and RAPA on the various parameters described, 30 allogeneic aorta transplantations were done (Tables 1 and 2). Treatment with high dose CsA or RAPA completely prevented the development of graft arteriosclerosis. Eight weeks after BN→WAG aortic transplantation, intimal thicknesses of 26 ± 13 μm and 56 ± 23 μm (P<0.001 and <0.01 vs. untreated controls) were found for CsA and RAPA, respectively. In the high dose CsA-treated allografts, even fewer intimal lesions were present than in the WAG autografts (P=0.05). In WAG→BN allografts, a significant reduction in intimal proliferation to 52 ± 46 μm and 32 ± 31 μm and P-values of <0.01 and <0.001 were found after high CsA and RAPA treatment 4 weeks after transplantation, respectively.

Only in the "weak" BN→WAG combination was treatment with low doses of CsA correlated significantly with a reduction in the severity of lesions. In this group, an intimal thickness of 75 ± 65 μm was measured, which was significantly less than in untreated controls (P<0.05). In comparison with the allogeneic controls, a significant increase in intimal thickness was seen in 2 of the low dose, CsA-treated WAG→BN aortic allografts (246 and 254 μm, P<0.05). In the remaining 2 allografts, the development of arteriosclerosis was inhibited (13 and 95 μm) In both experimental models, immunosuppression with high dose CsA and RAPA effectively reduced cellular infiltration. In these allografts, practically no adventitial inflammation was observed. Low dose CsA treatment was partially effective in inhibiting mononuclear cell infiltration. Severe rejection was observed in the 2 WAG→BN allografts in which increased intimal lesions had developed. Neither the medial thickness nor the amount of smooth muscle cell nuclei was affected significantly. Histology showed no damage to elastic fibers and an intact internal elastic lamina.

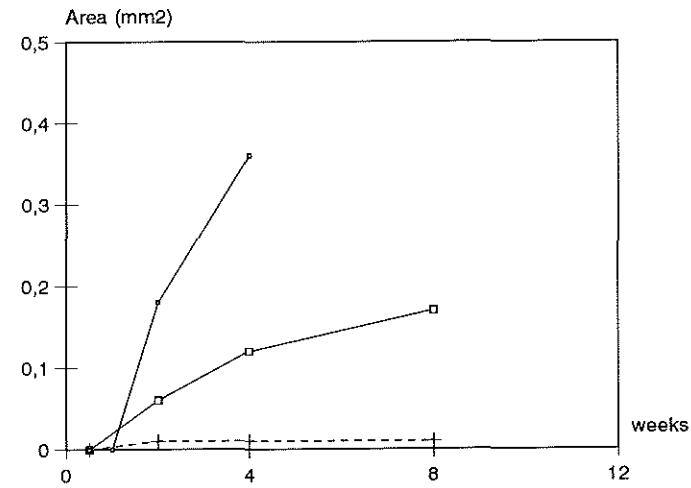

Figure 1. Intimal thickness. Numbers plotted are means. Solid lines represent aortic allografts and dashed lines represent syngeneic controls (small square = WAG→BN allografts, large square = BN→WAG allografts, X = WAG autografts, + = BN autografts).

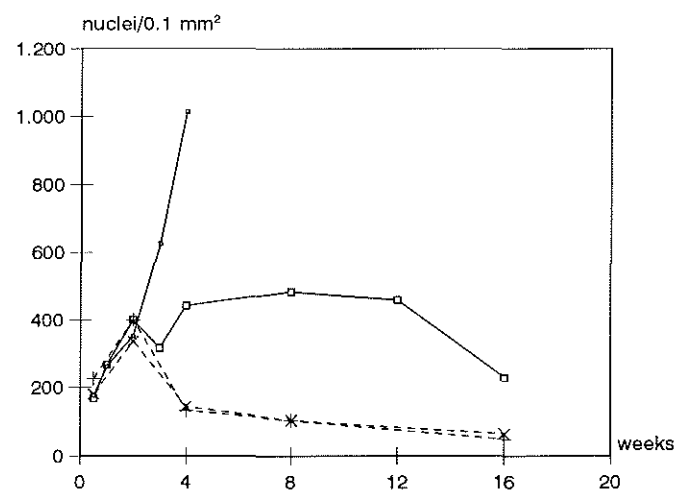

Figure 2. Adventitial nuclear density. Numbers plotted are means. For details of symbols, see legend to figure 1.

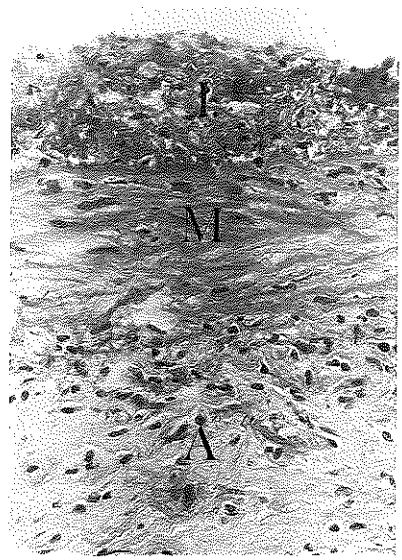

Figure 3. Transverse section of a BN→WAG aortic allograft 4 weeks after transplantation (I = intima, M = media, A = adventitia).

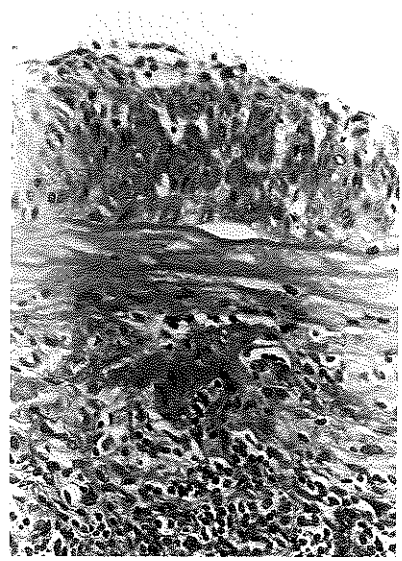

Figure 4. WAG→BN aortic allograft 4 weeks after transplantation. Note the severe adventitial infiltration in this "strong" WAG→BN combination as compared with the "weak" BN→WAG combination shown in figure 3.

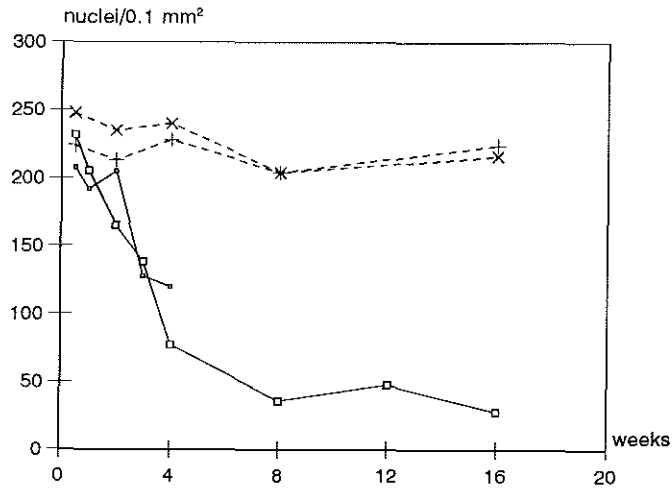

Figure 5. Medial nuclear density. Numbers plotted are means. Symbols as in fig.1.

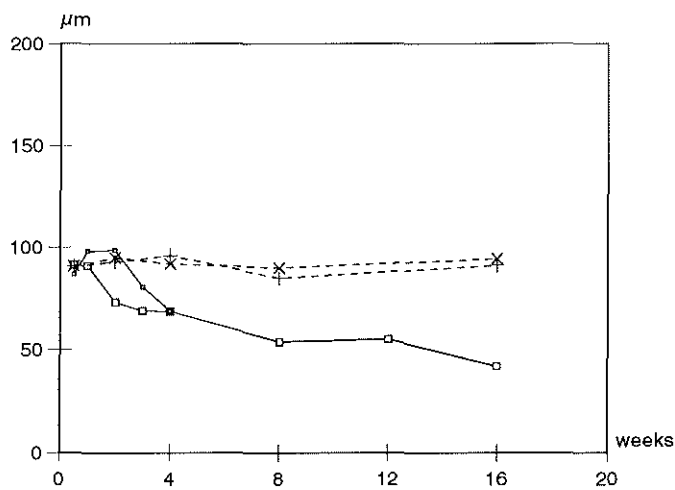

Figure 6. Medial thickness. Numbers plotted are means. For details of symbols, see legend to figure 1.

DISCUSSION

CsA has been reported previously to induce accelerated graft arteriosclerosis in the allogeneic aorta transplantation model 24). In both our aorta transplantation models, however, administration of high dose CsA effectively prevented graft arteriosclerosis. In high-dose-treated BN→WAG allografts, killed 8 weeks after transplantation, even fewer intimal lesions were present than in WAG autografts. These findings suggest that immunosuppression with CsA may prevent immune-mediated arteriosclerosis as well as the arteriosclerotic response to injury. Inhibition by CsA of smooth muscle proliferation in the vascular response to injury has been described previously.[25]

In the present study, increase of intimal thickness began shortly after initiation of acute rejection and followed this rejection response in the adventitia. This relationship between rejection and intimal proliferation suggests that cellular infiltration as an extent of acute rejection may result in arteriosclerosis. The denser adventitial mononuclear cell accumulations in the "strong" WAG→BN model may explain the more severe intimal proliferation in this combination. It is consistent with the hypothesis that graft arteriosclerosis is the result of an ongoing allogeneic immune reaction. In this hypothesis, cellular infiltration is likely to be involved by secretion of various cytokines and by direct cytotoxic effects.[26,27] The prevention of these lesions by CsA and RAPA may thus be explained by their inhibitory effect on acute rejection.

Increase of intimal thickness was seen in 2 WAG→BN allografts treated with low dose CsA. Adventitial infiltration was not prevented in these 2 cases, in contrast with the remaining 2 allografts in this group. Therefore, increased intimal proliferation may occur only after low ineffective CsA immunosuppression. Mennander, however, found that daily oral treatment with 5mg/kg CsA induced accelerated allograft arteriosclerosis in the DA→WF aorta transplantation model, whereas there was practically no cellular infiltration in the allograft adventitia.[24]

RAPA was as effective in preventing arteriosclerotic lesions as high dose CsA treatment. Previously, RAPA has been reported to prolong cardiac and renal allograft survival at doses as low as 0.08 mg/kg/day i.v.[28]. Higher doses of RAPA were needed to prevent arterial intimal thickening after allogeneic femoral artery transplantation. In this model, treatment of allograft recipients with 1.5 mg/kg/day i.v. was ineffective, whereas administration of 6mg/kg/day the

first week after transplantation, followed by 3 mg/kg/day reduced intimal thickening by 98%.[29] In experimental rat heart grafts, arteriosclerosis was effectively inhibited by 1.5 mg/kg/day RAPA.[30] In the present study, RAPA administration in a dose of 3mg/kg/3 times/week effectively prevented both acute rejection and arteriosclerotic alterations. Similarly, as in the CsA-treated groups, we found a correlation between acute rejection and graft arteriosclerosis. This finding suggests that the immunosuppressive effects of RAPA on graft arteriosclerosis, rather than its ability to inhibit non-immune-mediated arteriosclerosis, may be most important in this model.

Several rat heart transplantation models of chronic rejection have been described. In these models, graft arteriosclerosis developed after long-term, persistent, low level allograft rejection.[11,12] Immunosuppressive treatment with CsA, FK506, or azathioprine has been found to reduce the arteriosclerotic changes after allogeneic heart transplantation, whereas sensitization with skin grafts resulted in a more rapid onset of graft arteriosclerosis.[11,12,31] Apparently, in these heart models, the rejection grade is correlated with the degree of arteriosclerotic development. This is consistent with the severe myointimal proliferation in the present aortic allografts in strong, rejecting, nonimmunosuppressed recipients and prevention of this proliferation by CsA and RAPA.

Effective suppression of acute rejection prevented arteriosclerotic lesions in the present experimental aorta transplantation models. This suggests that chronic rejection may develop after ineffective suppression of acute rejection. This hypothesis is supported by several recent clinical studies showing a correlation between graft arteriosclerosis and the incidence of acute rejection episodes.[32-34] Clinical trials with high doses of immunosuppressants are unfortunately limited by the toxic side effects of various drugs, e.g., nephrotoxicity by CsA.[35] Studies on the efficacy of new immunosuppressive drugs with fewer toxic side effects or of potent synergistic drug combinations seem to be important to solve the problem of chronic rejection. The aorta transplantation model may, thereby, be useful in testing drugs or drug combinations.

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CHAPTER 6

KETANSERIN REDUCES GRAFT ARTERIOSCLEROSIS AFTER ALLOGENEIC AORTA TRANSPLANTATION IN THE RAT

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ABSTRACT

The serotonin-2 receptor antagonist ketanserin has been suggested to diminish arteriosclerotic development by its effect on platelet function and on vascular smooth muscle cells. We investigated the ability of ketanserin in reducing immune-mediated arteriosclerosis using the BN→WAG and WAG→BN rat aortic transplantation models.

Ketanserin (10 mg/kg/day), administered in drinking water significantly reduced posttransplant arteriosclerotic thickening of the intima in the BN→WAG model to 102 ± 23 μm as compared with 171 ± 60 μm in untreated BN→WAG allografts 8 weeks post-transplantation ($p<0.05$). In the opposite WAG→BN combination, at 4 weeks post-transplantation, no significant reduction in intimal thickening was attained (112 ± 42 vs. 152 ± 49 μm).

Platelet aggregation to increasing amounts of collagen did not show a correlation between the effect of ketanserin on platelet function and reduction in intimal thickening. Ketanserin had no effect on systolic blood pressure or mononuclear cell infiltration.

We conclude that ketanserin reduces graft arteriosclerosis by a mechanism other than by inhibition of platelet function, decrease in blood pressure or immunosuppression. Because of this antiarteriosclerotic effect, ketanserin therapy might be beneficial to the long-term survival of vascular allografts.

INTRODUCTION

With decreasing numbers of acute graft loss, chronic rejection has become the major complication of clinical transplantation. In heart grafts, chronic rejection is dominated by concentric intimal thickening of the epicardial as well as the intramyocardial branches of the coronary arteries. Because of this localization pattern, this phenomenon is also termed graft vascular disease (GVD). The prevalence of GVD ranges from 2 % to 18 % at 1 year and from 50% to 73% 5 years after transplantation.[1-4] This narrowing of the coronary arteries is the major limiting factor in medium- and long-term graft survival after heart transplantation. That GVD is a major problem even during the first year after transplantation, resulting in 33 and 68 % of patient death, has been reported by centers with long experience in heart transplantation.[5,6] There is no current treatment for chronic rejection, and in patients with

extensive GVD retransplantation remains the only option to prevent death.[7]

The pathogenesis of graft arteriosclerosis has not yet been elucidated but immunological phenomena linked to histoincompatibility differences between donor and recipient is generally believed to result in the development of proliferative lesions.[8,9] Repetitive endothelial injury with consequent platelet activation may thus contribute to the development of GVD.[10,11]

Studies of the mechanism of classic atherosclerosis have generated a resurgence of interest in serotonin. Serotonin is stored in the dense granules of platelets and when released, theoretically may stimulate thickening of the blood vessel wall in several ways, first in the positive feedback that it exerts on platelet aggregation. By amplifying the release of other aggregatory stimuli, serotonin will facilitate ongoing platelet aggregation and thus the release of growth factors, in particular platelet-derived growth factor (PDGF).[12,13] Second, serotonin has been demonstrated to stimulate directly the mitogenesis of aortic vascular smooth muscle cells (VSMC) in culture. Although substantially less potent in this respect than PDGF, serotonin in low concentrations significantly potentiates the VSMC mitogenesis of PDGF.[14] Last, serotonin may stimulate arteriosclerosis by its conceptive role in chronic hypertension.[15] Indeed, blocking the serotonin-2 ($5HT_2$) receptors by ketanserin, clinically applied as an anti-hypertensive agent, effectively antagonizes these effects of serotonin.[16-18] Therefore, ketanserin has been suggested to provide protection to arteriosclerotic diseases.[19]

In previous immunohistologic studies of graft arteriosclerosis in aortic allografts we observed predominantly actin-positive VSMC in late intimal lesions. Furthermore, we observed more severe arteriosclerotic alterations in the WAG→BN rat strain combination than in the reverse combination.[20] Because of our results showing that the BN has a stronger blood-clotting tendency than the WAG rat, in the present study we examined the role of platelet aggregation in the development of posttransplant arteriosclerotic lesions. We also tested the hypothesis that ketanserin provides vascular protection by affecting platelet function and by blocking directly the growth stimulation of VSMC by serotonin.

The effects of ketanserin on blood pressure and on collagen induced platelet aggregation were monitored.

MATERIALS AND METHODS

Experimental animals

Male inbred Brown Norway (BN, RT1^n) and WAG (RT1^u) rats were used. All animals were obtained from Harlan CPB, Austerlitz, The Netherlands and had free access to food and water. Rats of both strains, weighing 200-250 g and aged 10-12 weeks, were used as recipients and donors. The experimental protocols were approved by the Committee on Animal Research of Erasmus University, Rotterdam, the Netherlands, and adhered to the *Guidelines on the Protection of Experimental Animals* of the Council of the EC (1986).

Aorta transplantation

All rats were anesthetized with ether and underwent laparotomy. In the donor as well as the recipient, a segment of infrarenal aorta, approximately 1 cm long, was isolated, excised, perfused with saline, and used as a transplant. Donor aorta was transplanted into orthotopic position. End-to-end anastomosis was performed with a 9.0 monofilament nylon suture (Ethicon, Sommerville, N.J, U.S.A). The ischemic time was 20-30 minutes.

Ketanserin treatment

Ketanserin (Ketanserin-tartrate, Janssen, Beerse, Belgium) was given orally in a daily dose of 10 mg/kg, dissolved in drinking water. Although this dose of ketanserin is high as compared with human standards, no toxic side-effects were noted in a pilot study. Ketanserin was dissolved in drinking water to maintain constant blood level. To prevent precipitation, a freshly prepared ketanserin solution was provided daily. We ascertained intake of 10 mg/kg ketanserin by each animal by measuring their daily water consumption.

Experimental design

In a previous study, we observed an increase in intimal thickness in the BN→WAG and WAG→BN aortic transplantation models as well as in BN and WAG aortic autotransplants.[20] In the WAG→BN model the process of intimal thickening was so rapid that severe lesions had already developed 4 weeks post-transplantation. Due to intense intimal thickening, thrombosis and necrosis, all allografts of this strain combination removed at later timepoints were not

useful for evaluation. In the reverse combination (from the BN→WAG), intimal lesions developed more slowly. In this rat combination, a steady increase to severe lesions at 8 weeks was evident. Therefore, in this study, animals in the WAG→BN combination were killed at 4 weeks and those in the BN→WAG combination were killed at 8 weeks. The experiment was performed with four groups of rats. The different groups with corresponding treatments and numbers of the animals are shown in table 1.

Histology

Straight 5-μm cross-sections from tissues embedded in paraffin, were prepared at three levels of the midportion of the graft and stained with hematoxylin-eosin and with elastic of Gieson. Slides were then examined by light microscopy. The thickness was measured with a calibrated ocular micrometer to evaluate the following variables: the intimal and medial thickness, adventitial infiltration, and VSMC necrosis. The average medial thickness and maximal intimal thickness were determined. Cellularity of the adventitia, as a measure of cellular infiltration, and of the media was assessed by counting the number of nuclei at 5 sites. The mean score was multiplied to a field of 0.1 mm^2.

In vitro platelet aggregation assays

Platelet function was measured at the times the animals were killed: at 4 weeks post-transplantation in the WAG→BN model and at 8 weeks in the BN→WAG model. Blood from all animals receiving ketanserin and blood from 6 untreated transplanted BN and WAG rats each, as controls, were analyzed. In addition, platelet function of 3 BN and 3 WAG rats was tested 3 h after administration of a high dose of ketanserin by gastric intubation (50 mg/kg) determine whether the dose of 10 mg/kg reduced platelet aggregation in the experimental animals. Under ether anesthesia, the animals received 50 IU heparin intravenously, after which the abdomen was opened. At the time of death, the abdominal aorta was dissected at its bifurcation, and blood was collected by aortic puncture. Platelets were aggregated with the Chronolog-Whole Blood Aggrometer (Chronolog U.K.). Collagen was chosen to induce platelet aggregation, because of its likely physiological role in mediating platelet aggregation in vascular allografts. Increasing concentrations of 0.025 to 2.50 mg/ml collagen were added to heparinized (10 U/ml) whole blood samples, which were diluted 1:1 with 0.9% saline. Ten-

minute recordings of the induced changes in electrical resistance (impedance, in Ω) were analyzed by an Olivetti M24 PC. The amount of platelets was counted on a TOA platelet counter PL100.

Measurement of blood pressure

The effect of ketanserin on systolic blood pressure was measured in conscious rats by the tail-cuff method with a electrosphygmomanometer (Narco Bio-Systems, Houston, TX). Measurements were made in both BN→WAG and WAG→BN transplanted rats receiving ketanserin as well as in the untreated transplanted control rats 1 week before and 0.5, 1 and 2 weeks after transplantation.

Statistical analyses

The results are mean and standard deviation (S.D.). The data were assessed statistically by analysis of variance (ANOVA) followed by a Bonferroni *t* test; $p < 0.05$ was considered statistically significant.

RESULTS

Effect of ketanserin on graft arteriosclerosis

As shown in table 1, ketanserin significantly reduced intimal thickening in the BN→WAG combination to 102 ± 23 μm, compared with 171 ± 60 μm in the allogeneic controls 8 weeks after transplantation ($p<0.05$). In the WAG→BN combination, no significant inhibition was observed 4 weeks after transplantation (121 ± 64 vs. 152 ± 49 μm). Ketanserin treatment did not affect adventitial cellular infiltration or VSMC necrosis. No toxic side effects of ketanserin were noted during the experimental period.

Table 1. Effects of ketanserin treatment on the intima, media, and adventitia in the BN to WAG and WAG to BN models.

Group	Strain combination	n	Ketanserin treatment	week	Intima thickness	Media thickness	Media nuclei	Adventitia nuclei
1	BN-WAG	9	-	8	171±60	54±7	36±22	484±188
2	WAG-BN	10	-	4	152±49	69±5	120±40	1014±520
3	BN-WAG	6	10mg/kg	8	102±23*	47±13	53±26	554±135
4	WAG-BN	6	10mg/kg	4	112±42	75±8	132±24	924±356

Results are presented as mean ± S.D. The intimal and medial thicknesses are expressed in micrometers; the numbers of nuclei in the media and adventitia in thickness are multiplied to a field of 0.1 mm^2.

*P<0.05 versus group 1.

Platelet aggregation

There was no difference in number of platelets between the BN and WAG rat strains. Changes in electrical resistance as a result of collagen-induced platelet aggregation in the different experimental groups are shown in figures 1 to 3. Untreated BN platelets were more sensitive to collagen than WAG platelets. Collagen in a concentration as low as 0.050 mg/ml was effective in inducing BN platelet aggregation. A concentration of 0.200 mg/ml decreased electrical resistance by 1.57 Ω, whereas untreated WAG platelets were not affected at this concentration. Aggregation of WAG platelets was observed only at collagen concentrations ≥0.250 mg/ml. After ketanserin treatment, BN platelet aggregation was observed at collagen concentrations ≥200mg/ml, showing an aggregation pattern similar to that of untreated WAG platelets. WAG platelet function at low collagen concentrations was not altered by ketanserin treatment. At high concentrations of collagen, no difference in electric resistance was observed in either rat strains as compared with the untreated transplanted controls. Similar patterns of collagen-induced platelet aggregation were noted 3 h after high-dose ketanserin (50mg/kg) administration to normal rats of the two different strains (Fig. 3). This finding supports a maximal therapeutic effect of 10 mg/kg ketanserin as administered to the experimental animals.

Effect of ketanserin on systolic blood pressure

Before transplantation, BN rats had systolic blood pressure of 125 ± 6 mm HG as compared with 114 ± 7 mm HG in untransplanted WAG rats. After BN→WAG and WAG→BN aortic transplantation, systolic blood pressure did not change in untreated controls (115 ± 7 and 121 ± 7 mm HG at day 7 and 117 ± 8 and 126 ± 9 mm HG at day 14 post-transplantation, respectively) or after ketanserin treatment (114 ± 7 and 128 ± 9 mm HG at day 7 and 116 ± 9 and 132 ± 8 mm HG at day 14).

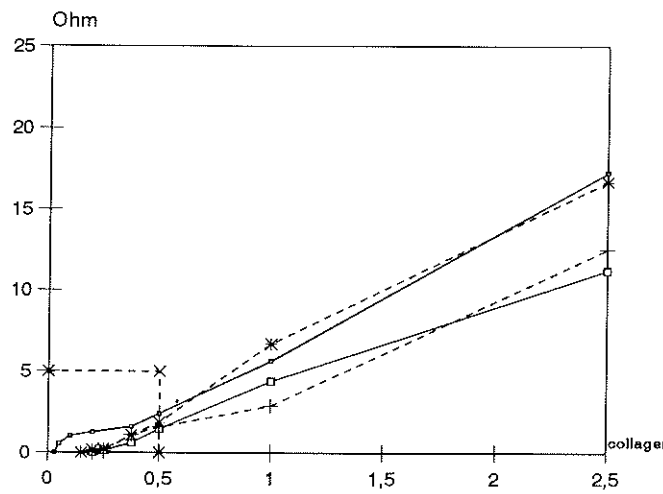

Figure 1. Changes in electrical resistance in ohms as a result of platelet aggregation to incremental amounts of collagen in blood derived from the experimental animals. Untreated animals (solid lines); ketanserin-treated animals (dotted lines). WAG→BN transplanted animals (open squares); BN→WAG transplanted animals (X).

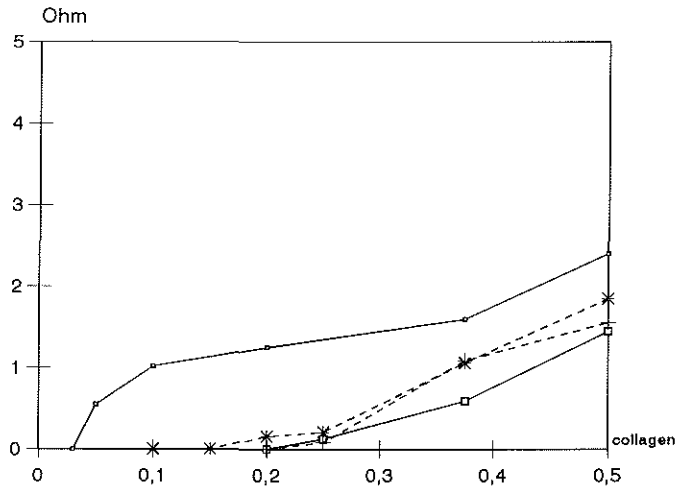

Figure 2. Magnification of portion of Fig. 1. for lower concentrations of collagen. For details of symbols, see legend to figure 1.

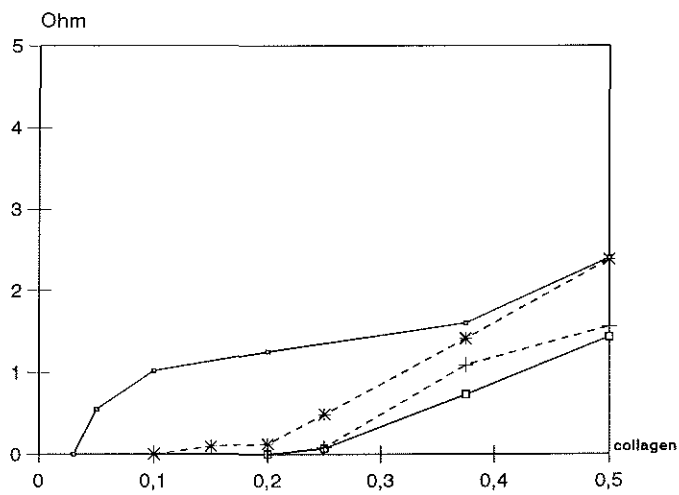

Figure 3. Changes in electrical resistance in ohms as a result of platelet aggregation to incremental amounts of collagen in blood from normal animals 3 hours after high-dose ketanserin (50 mg/kg) administration. Untreated animals (solid lines); ketanserin-treated animals (dotted lines). BN rats (open squares); WAG rats (X).

DISCUSSION

Ketanserin has been suggested to provide vascular protection by blocking the effects of serotonin on platelets and VSMC.[19] Indeed, in the present study, the development of posttransplant arteriosclerosis was significantly reduced in the BN→WAG combination. The ability of ketanserin to block $5HT_2$ receptors therefore suggests that platelet-derived serotonin might be involved in the mechanism leading to posttransplant arteriosclerosis. Serotonin mediated platelet aggregation has been demonstrated to be reduced by ketanserin.[16,17] In the present study, the sensitivity of BN platelets was also reduced to low concentrations of collagen after ketanserin treatment. The inhibiting effect of ketanserin on BN platelet aggregation, did not coincide with significant inhibition of the arteriosclerotic response in the WAG→BN aorta transplantation model, however. Similarly, in isografts a greater sensitivity of BN platelets did not result in more pronounced arteriosclerotic lesions in BN rats than in WAG rats.[20] On the other hand, intimal thickening was significantly reduced in the BN→WAG model, in which no effect of ketanserin on collagen-induced platelet aggregation could be detected. Therefore, we noted no correlation between the effect of ketanserin on platelet function and reduction of intimal thickening, which suggests that ketanserin might reduce arteriosclerosis by a mechanism other than by affecting platelet function.

A direct blocking effect by ketanserin on the serotonin-induced mitogenesis of VSMC is a likely explanation for the antiproliferative effect. In vitro, serotonin has been reported to stimulate the mitogenesis of aortic VSMC.[14] Any immunosuppressive effect of ketanserin is unlikely because no differences in mononuclear cell infiltration were evident between ketanserin-treated and untreated allografts in the present study.

The significant reduction in intimal proliferation induced by ketanserin in the BN→WAG aortic model suggests that the stimulating effects of platelet-derived serotonin on the mitogenesis of VSMC might be of relatively greater importance in the BN→WAG model than in the WAG→BN model, possibly owing to differences in mononuclear cell infiltration in the two rat strain combinations.[20] More severe cellular rejection in the WAG→BN combination may indicate a minor role of serotonin in the overall arteriosclerotic stimulation after aorta transplantation. On the contrary, relatively weaker rejection in the BN→WAG combination may provide local conditions that cause serotonin to be of significant importance in stimulating

VSMC proliferation.

Ketanserin did not affect blood pressure during the experiment. This finding is in agreement with results of previous studies that showed a minor and brief response of blood pressure to keatanserin in conscious normotensive animals as compared with anesthetized animals or conscious hypertensive rats.[21,22] The hypotensive effect of ketanserin therefore can be excluded as a mechanism leading to decreased graft arteriosclerosis.

Serotonin has been suggested to be a mediator of cyclic flow alterations in stenosed coronary arteries in dog studies. These flow variations are believed to be produced by platelet aggregation at the stenotic site alternating with thrombus dislodgement and embolization. The frequency and severity of cyclic flow alterations and platelet aggregation predict the severity of neointimal proliferation. Ketanserin treatment was successful in abolishing these blood flow variations and in retarding neointimal proliferation.[23,24] Our study showed a decrease in BN platelet sensitivity to low concentrations of collagen with no effect on maximal platelet aggregation. Therefore, an increase in the threshold of platelet activation by ketanserin treatment might explain the mechanism of alternating cyclic flow variations.[24] This hypothesis is also in accord with the reported effect of ketanserin in prolonging rat tail bleeding time.[25] Used clinically, ketanserin causes a greater decrease in blood pressure in hypertensive or older patients.[21,22,26] Similarly, ketanserin causes a considerably greater reduction in serotonin-induced platelet aggregation in older than in younger patients.[27] These findings suggest that ketanserin might be more effective in reducing posttransplant arteriosclerosis when serotonin is of greater importance in the associated hemodynamic processes. A similar explanation was offered in a large clinical study of restenosis after coronary angioplasty in which no protective effect by ketanserin was detected. The lack of effect in this trial may indicate a minor role of serotonin in the complex biology of late restenosis.[28] Future studies of ketanserin in older, hypertensive, or partially immunosuppressed animals may demonstrate its effects in reducing posttransplant arteriosclerosis.

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CHAPTER 7

GENERAL DISCUSSION, CONCLUSIONS AND FUTURE PERSPECTIVES

INTRODUCTION

In this chapter we return to the basic questions that underlie the studies presented in chapter 2-6. We will discuss the findings of these studies concerning the pathogenesis and therapeutic approaches of chronic rejection. As described in chapter 1, chronic rejection is the leading cause of long-term failure of heart transplants as the result of concentric intimal thickening diffusely involving the coronary arterial tree.[1-4] At present, the knowledge on the mechanism and treatment of chronic rejection is unsatisfactory, and therefore retransplantation remains the only effective therapy.[5]

The aorta transplantation model and graft arteriosclerosis

Heart transplantation models in rats are frequently used in the study of chronic rejection.[6-8] However, there are important disadvantages linked to these models. To prevent graft loss due to acute rejection, allotransplants are performed in immunological weak strain combinations or under immunosuppression. In these settings of low-grade graft rejection, chronic rejection first appears several months post-transplantation, which makes heart transplantation models time-consuming. Aortic allografts however, are not lost due to acute rejection and therefore do not require immunosuppression. These non-immunosuppressed aortic grafts start to develop intimal lesions within 2 weeks, which makes this model time-efficient and less expensive. An additional advantage of the aortic transplantation model is the possibility to investigate the effects of individual drugs on graft arteriosclerosis without being forced to give any immunosuppressive agent. Williams et al. were the first in 1975 to study the rat aorta transplantation model.[9] In 1990, Halttunen et al. re-introduced the aortic transplantation model as an experimental model of chronic rejection. They showed that similar pathogenetic events take place as in human heart transplants suffering from chronic rejection.[10,11] Nowadays, the aorta transplantation model has proven to be a useful tool in the study of chronic rejection.

Platelet function and graft arteriosclerosis

Ross proposed in the mid-1970s the "response-to injury" hypothesis to explain the

pathophysiology of chronic rejection.[12] According to this hypothesis the primary lesion is loss of the vascular endothelial cell lining, followed by platelet adhesion. The subsequent release of platelet products would stimulate VSMC to proliferate and migrate into the intima. However, this hypothesis is under debate because the endothelium has been shown to remain intact while the vessel wall undergoes arteriosclerotic transformation.[13] In chapter 6 we studied the effect of platelet function on the development of graft arteriosclerosis using the aorta transplantation model. In this model we found no evidence that platelet function might be involved in the development of chronic rejection which is in support of other studies on the anti-platelet drugs aspirin and dipyridamol. These studies also found that platelet function is not related to the development of graft arteriosclerosis.[14,15]

The pathogenesis of graft arteriosclerosis

The pathophysiology of chronic rejection is complex and multifactorial. There is still no generally accepted theory on the pathogenesis of chronic rejection. Although alloantigen-dependent factors likely play a central role in the development of graft arteriosclerosis, it is still controversial whether humoral or cellular immunity predominate.[16,17] In chapter 2 we characterized the immunopathologic events leading to graft arteriosclerosis in the aorta transplantation model. In this model, intimal lesion development was preceded by deposition of IgM antibody and complement in the aortic wall. Progressive intimal lesions were characterized by increasing numbers of infiltrating cells. Therefore, in the aorta transplantation model, humoral rejection seems to play a role in the initiation of intimal lesions, while cellular rejection appeared to be responsible for its progression. That both humoral and cellular rejection are required for the development of graft arteriosclerosis, has recently been demonstrated in carotid artery vascular grafts in mice with immunological defects. This study showed that CD4+ T cells, humoral antibody and macrophages are essential for the development of intimal lesions in contrast to CD8+ T cells and natural killer cells.[18]

Cytokines and graft arteriosclerosis

Cytokines are known to play an important role in acute graft rejection.[19-22] In chronically

rejecting heart and kidney transplants however, it is, from a technical point of view, difficult to study cytokine gene expression. For this reason, little is known about the role of cytokines in chronic rejection. A likely role for cytokines in chronic rejection however, has been suggested by many authors.[23-31] In chapter 4 we investigated whether cytokines play a role in graft arteriosclerosis. In rat aortic allografts, detectable cytokine mRNA expression for IL-1β, IL-2, IL-6 and IFN-γ was found. In the LEW to BN combination, which develops intimal lesions more rapidly, these mRNA expressions remained detectable after 2 weeks post-transplantation, whereas the expressions in the "weak" BN to LEW combination disappeared. These results provide evidence for local vascular expression of cytokine mRNA and suggest that cytokines play a role in the process of graft arteriosclerosis.

Cyclosporine and graft arteriosclerosis

Cyclosporin (CsA) very effectively reduces early graft loss from acute rejection, but in time, chronic rejection occurs in the majority of grafts.[32] The lack of efficacy of CsA to prevent chronic rejection has been suggested to be related to its preferential effect on the T-helper-1 subset of CD4+ cells. Because of a small effect on the T-helper-2 cells, CsA would fail to suppress cytokine production that helps B cells to develop into plasma cells.[33] Mennander et al. even found that low dose CsA could induce transplant arteriosclerosis by inducing "endothelialitis" and suggested that CsA damages the endothelial cell lining of the allograft.[34] In chapter 5 however, we showed that effective immunosuppression by CsA prevents graft arteriosclerosis. We did not find CsA to be toxic for the endothelium or to cause endothelialitis. Stoltenberg et al., who even found effective inhibition of graft arteriosclerosis with low-dose CsA treatment, confirmed this.[35-37]

Acute rejection and graft arteriosclerosis

In chapter 5 we found that effective suppression of acute vascular rejection prevents graft arteriosclerosis. This finding suggests that chronic rejection may develop after ineffective suppression of acute rejection. Clinically, this hypothesis is supported by several studies showing a correlation between graft arteriosclerosis and the incidence of acute rejection

episodes.[38-40] This could explain why only few differences can be found between infiltrating cell populations of acutely rejecting, chronically rejecting or well-functioning allografts.[41,42] Therefore, to prevent graft arteriosclerosis, one should try to sustain an optimal level of immunosuppression. Clinical trials with high-doses of immunosuppressive agents are unfortunately limited by the toxic side effects of various drugs, e.g. nephrotoxicity by CsA.[43] Studies on the efficacy of new immunosuppressive drugs with less toxic side effects or on potent synergistic drug combinations are clearly important to solve the problem of chronic rejection.

Ischemia-reperfusion and graft arteriosclerosis

Although ischemia has long been considered to be an alloantigen-independent risk factor in chronic rejection, the contribution to this process is still unclear.[44] Some clinical studies showed a positive influence of ischemia on chronic rejection, whereas others found no correlation.45-48 Likewise, experimental studies are inconsistent with regard to the role of ischemia in graft arteriosclerosis.[49-51]

Several mechanisms of injury from ischemia and reperfusion have been suggested. Endothelial cell injury may occur with increased expression of the growth factor PDGF, adhesion molecules, and MHC antigens, which may result in increased graft immunogenicity.[52-54] Subsequently, increased graft immunogenicity may explain the correlation between ischemic time and the number of acute rejection episodes.[55] In chapter 5 we looked in the aorta transplantation model for a possible association between ischemia-reperfusion and graft arteriosclerosis. After 3 hr of ischemia-reperfusion, focal deposits of IgM antibody and complement membrane attack complex (C5b-9) were observed in both aortic isografts and allografts. The pattern of these IgM and C5b-9 deposits resembled the immunological findings in aortas with early intimal lesions as reported in chapter 2. Therefore, these observations suggest that IgM deposition and complement activation in the early reperfusion period play a role in transplant arteriosclerosis.

CONCLUSIONS

1 The aorta transplantation model is a useful tool in the study of chronic rejection.

2 IgM antibodies and complement are involved early in the development of intimal lesions.

3 Ischemic injury is characterized by IgM antibody and complement depositions. This suggests that ischemia plays a role in the development of graft arteriosclerosis.

4 In aortic allografts, cytokine gene expression for IL-6, IL-1β, IL-2 and IFN-γ was found, which are correlated to the progression of graft arteriosclerosis.

5 Suppression of acute rejection prevents graft arteriosclerosis.

6 Platelet-function is not correlated with graft arteriosclerosis in the aorta transplantation model.

7 Ketanserin reduces graft arteriosclerosis, likely by blocking serotonin induced mitogenesis of VSMC.

CURRENT AND FUTURE PESPECTIVES

With the introduction of cyclosporin (CsA) in the early 1980s, transplantation has become a routine procedure in medicine. Recently, its absorption has been improved by the introduction of Neoral, the new formulation of CsA, which gives significantly better and more consistent absorption.[56] The efficacy and safety of Neoral has been confirmed in large clinical studies.[57,58] Tacrolimus (FK 506), a new immunosuppressive agent, was used first by the University of Pittsburgh.[59] Clinically, its efficacy in the prevention of acute rejection is comparable to that of CsA.[60] However, depending on the clinical side-effects of CsA, conversion to FK506 treatment can be decided on an individual basis.[61]

Rapamycin (RAPA) is another new potent immunosuppressant. In combination with Neoral, a reduction of acute rejection episodes of about 30% is achieved.[62] Ongoing large Phase III randomized double-blinded trials are examining the benefit-to-risk ratio of RAPA therapy in clinical transplantation.[63] Recently, an oral formulation of RAPA, SDZ RAD, has become available. Initial studies showed this medicine to be safe. In the rat aorta transplantation model, SDZ RAD significantly reduced the development of chronic rejection, which may have clinical implication for long-term graft survival.[64]

There is increasing evidence that acute rejection is a significant risk factor for the development of chronic rejection.[65] Thereby, a relationship can be found between the time of the occurrence of acute rejection and the development of transplant arteriosclerosis.[65] Acute rejection episodes occurring late after transplantation appear to be associated with a higher risk for the development of chronic rejection than rejections occurring early after transplantation.[66-68] In addition, there seems to be a relationship between the severity of acute rejection episodes and the development of chronic rejection. Vereerstraeten et al. showed that only severe acute rejection episodes increase the risk on chronic rejection, whereas mild rejections with complete functional recovery of the graft do not have a deleterious effect on long-term outcome.[69] Therefore, prevention of acute rejection may be one of the most important tools to diminish the incidence of chronic rejection in the future. This importance is illustrated by a recent clinical study of Kliem et al. who found enhanced immunosuppression, by conversion from a double-drug regimen with CsA/prednisolone to a triple-drug regimen including azathioprine, a successful therapy of chronic renal allograft failure.[70] In up to 75% of the patients,

conversion to this enhanced immunosuppression therapy was followed by a significant improvement in graft function.

Another approach to decrease the incidence of chronic rejection may be the induction of tolerance in the recipient. If donor-specific unresponsiveness can be created, it may be possible to reduce patients' conventional immunosuppression therapy. Induction of allospecific-tolerance has been achieved in animal models, like the aorta transplantation model.[71,72] Investigations are underway into obtaining tolerance by mixed chimerism, a process wherein donor bone marrow is infused during transplantation.[73]

Both the short and the long-term success rates of transplanted organs from brain-dead donors are consistently inferior to those of living sources.[74] As cadaver and living unrelated donors are equally genetically disparate with a given recipient, the difference in results could relate to circumstances peculiar to the donor and to changes associated with ischemia-reperfusion injury.[75] In an experimental model of brain death in rats, mRNA expression in kidneys of IL-1, IL-6, TNF-α and IFN-γ was induced 6 hr after induction of brain death.[76] Immunohistochemistry showed an increase in numbers of intrarenal leukocytes and upregulation of P- and E-selectin expression as well as of MHC class I and II antigens. The activation of peripheral organs after induction of brain death is thought to be caused by various interrelated events like hypotension and ischemia.

Ischemia is thought to increase the incidence of chronic rejection by increasing the rate of acute cellular rejection.[52-55,77] If ischemia indeed initiates chronic rejection by stimulating acute rejection, than treatment of ischemia-reperfusion injury may increase long-term graft survival. This hypothesis was recently tested by the group of Tilney, who used a soluble P-selectin glycoprotein ligand (sPSGL) to inhibit ischemia reperfusion injury.[78] Although this therapy inhibits acute rejection in the allogeneic situation, its effect on chronic rejection still has to be proved. In a syngeneic situation however, sPSGL treatment was able to prevent the development of ischemia induced late renal changes with decrease in graft function.[79]

IFN-γ has already in 1989 been postulated by Libby et al. to play a central role in chronic rejection because of its effect on T cells, macrophages and NK cells.[80] Recently, the availability of IFN-γ-deficient mice permitted this group to test critically the contribution of IFN-γ to the development of chronic rejection.[81,82] Cardiac allografts in IFN-γ-deficient mice developed only minimal or no transplant arteriosclerosis as compared with controls. In addition, similar results on graft arteriosclerosis were found after administration of IFN-γ

neutralizing antibody in normal rats. These data establish IFN-γ as a critical factor in the pathogenesis of chronic rejection and therefore, anti-IFN-γ antibody treatment may, in the future, become a feasible approach to improve long-term graft survival.

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CHAPTER 8

SUMMARY

CHAPTER 1: Introduction.

Progress in immunosuppressive therapy has improved the prevention and management of acute rejection in cardiac transplantation. Consequently, the development of a distinct form of coronary arteriosclerosis in the arteries of heart transplants, also called chronic rejection, has emerged as a major problem to long-term graft survival. Nowadays this phenomenon of chronic rejection has been reported to be a major cause of long-term morbidity and mortality after cardiac transplantation. Chronic rejection is not only restricted to heart transplants, but has also been described in kidney, liver, bowel and lung transplants. There is still no treatment to prevent graft arteriosclerosis and retransplantation remains the only effective therapy.
The pathogenesis of graft arteriosclerosis has not yet been elucidated, but the general believe is that immunological phenomena linked to histocompatibility differences between donor and recipient finally result in the development of proliferative intimal lesions.
To study the pathogenesis of chronic rejection, we introduced the rat aorta transplantation model into our laboratory. In this model, graft arteriosclerosis develops within 3 weeks post-transplantation, which makes this model a useful tool for experimental studies.
The aim of this thesis was to investigate the pathogenesis and therapeutic approaches of chronic rejection.

CHAPTER 2: Accelerated arteriosclerosis in aortic grafts: a role for activated complement and IgM in early lesion development.

The immunopathologic events underlying chronic rejection were studied in the aorta transplantation model. 19% of the LEW→LEW and BN→BN isografts contained small intimal lesions. After the second week post-transplantation, no growth of these intimal lesions was observed. At 2 weeks, in 22% of the LEW→BN and BN→LEW allografts intimal lesions were seen, increasing to 87% at 8 weeks. Immunohistochemistry of the isografts showed only weak staining of IgM and C5b-9 in the intimal layer. In contrast, in all allografts with intimal hyperplasia, dense IgM and C5b-9 staining was seen in intimal and medial smooth muscle layers. In early intimal lesions these IgM and C5b-9 depositions were found to be infiltrated mainly by macrophages. This finding suggests that the functional significance of complement

in graft arteriosclerosis may depend to a large degree upon its effects on macrophages. Activated complement has been described to promote the binding and infiltration of macrophages to the vessel wall. It also stimulates several macrophage functions, particularly the release of IL-1 which can increase vascular smooth muscle cell (VSMC) proliferation in vitro, promote endothelial cell adhesiveness for leukocytes and upregulate the adhesive protein ICAM-1.

This study also showed induced expression of endothelial ICAM-1 and MHC class II in both aortic isografts and allografts. Induction of ICAM-1 and MHC class II expression is considered to play a role in the modulation and maintenance of vascular inflammation by their effect on leukocytes. In isografts, temporal vascular inflammation may result in the development of self-limiting intimal hyperplasia, whereas persistent vascular inflammation in allografts may result in progressive graft arteriosclerosis characteristic of chronic rejection.

CHAPTER 3: Immunopathological events early after aorta transplantation: a study on ischemic injury.

Studies have shown that both alloantigen dependent and independent factors may be involved in the development of chronic rejection. However, it is still unclear if ischemia contributes to this process as well. Some clinical studies showed a positive influence of ischemia on chronic rejection, whereas other studies found no correlation. Experiments in animals also show little consistency with regard to the role of ischemia in graft arteriosclerosis. Therefore, in this study, we looked for a possible correlation between graft arteriosclerosis and ischemic injury in the aorta transplantation model. To induce ischemic injury, donor aortas were stored for 1 hour at room temperature in tris buffered saline before transplantation into the recipient. Animals were sacrificed 3 hours after reperfusion and tissues were processed for immunohistochemistry. In both isografts and allografts, focal depositions of IgM antibody and C5b-9 were found in the subintimal and medial layers. The pattern of these IgM and C5b-9 deposits resembled those found in early graft arteriosclerosis as showed in chapter 2. This observation suggests that ischemic injury may stimulate the development of graft arteriosclerosis by activation of the complement cascade and that the development of graft arteriosclerosis may be reduced by inhibition of the complement system.

CHAPTER 4: Accelerated arteriosclerosis in aortic grafts: a role for cytokines in progressive intimal lesion development.

A relationship between vascular mRNA cytokine expression and the severity of arteriosclerosis in the aortic transplantation model was demonstrated. Cytokine mRNA profiles revealed significantly increased mRNA for IL-1β, IL-6, IFN-γ, IL-2 and TNF-α in aortic allografts. This cytokine gene expression was noted only in the early post-transplant sacrifice timepoint in the "weak" BN→LEW combination, whereas persistent mRNA levels were found in LEW→BN allografts with progressive intimal lesions. In aortic isografts only mRNA gene expression for TNF-α and TGF-β1 was significantly increased after respectively 2 and 3 weeks. These findings provide evidence for increased local expression of cytokine mRNA in post-aortic transplant arteriosclerosis and suggests that local cytokine release may play a predominant role in determining the severity of intimal proliferation.

CHAPTER 5: Suppression of acute rejection prevents graft arteriosclerosis after allogeneic aorta transplantation in the rat.

The development of graft arteriosclerosis was found to be related to (acute) vascular rejection. Effective immunosuppression by cyclosporine (CsA) and rapamycin inhibited the formation of intimal thickening. In high dose CsA treated BN→WAG allografts, even less graft arteriosclerosis was present than after syngeneic aorta transplantation in de WAG without CsA treatment. This finding suggests that effective immunosuppression with CsA may prevent immune mediated arteriosclerosis as well as the arteriosclerotic response to injury. Treatment with low doses of CsA was only in the BN→WAG combination significantly correlated with a reduction in the severity of lesions. In the opposite WAG→BN combination, low dose treatment with CsA did not result in significant inhibition of graft arteriosclerosis. Because of these findings we came to the hypothesis that chronic rejection in humans may develop after ineffective suppression of acute rejection. This is supported by several clinical studies showing a correlation between graft arteriosclerosis and the incidence of acute rejection episodes. Unfortunately, clinical trials with high-doses of immunosuppressive agents are limited due to the toxic side effects of various drugs, e.g. nephrotoxicity by CsA.

CHAPTER 6: Ketanserin reduces graft arteriosclerosis after allogeneic aorta transplantation in the rat.

The serotonin-2 receptor antagonist ketanserin has been suggested to diminish arteriosclerotic development by its effect on platelet function and on vascular smooth muscle cells. We investigated the ability of ketanserin to reduce immune-mediated arteriosclerosis using the rat aorta transplantation model. Ketanserin (10 mg/kg/day) significantly reduced posttransplant arteriosclerotic thickening of the intima in the BN→WAG model. To determine the mechanism by which ketanserin reduced intimal hyperplasia, its effect on collagen induced platelet aggregation was studied. Platelets from untreated BN animals were more sensitive to collagen than platelets from WAG rats. In the present study, the sensitivity of BN platelets was reduced after ketanserin treatment. However, the inhibiting effect of ketanserin on BN platelet aggregation did not coincide with significant inhibition of the arteriosclerotic response in the WAG→BN aorta transplantation model. On the other hand, intimal thickening was significantly reduced in the BN→WAG model, in which no effect of ketanserin on collagen-induced platelet aggregation could be detected. So, in this study we found no correlation between the effect of ketanserin on platelet function and reduction of intimal thickening, which suggests that ketanserin might reduce arteriosclerosis by a mechanism other than by affecting platelet-function. A direct blocking effect by ketanserin on the serotonin induced mitogenesis of VSMC is a likely explanation for the antiproliferative effect, since serotonin has been reported to stimulate the mitogenesis of aortic VSMC in vitro. The mild inhibition of graft arteriosclerosis by ketanserin in this study may indicate a minor role of serotonin in the complex mechanism leading to graft arteriosclerosis.

CHAPTER 9

NEDERLANDSE SAMENVATTING

HOOFDSTUK 1: Introductie.

De ontwikkelingen in de immunosuppressieve therapie hebben geleid tot een verbeterde behandeling en preventie van acute afstoting na harttransplantaties. Door de verlengde transplantaat overleving openbaart zich echter een nieuw probleem. In de harttransplantaten ontstaat arteriosclerosis in de kransslagaders. Dit proces wordt ook chronische afstoting genoemd.

Op dit moment is chronische afstoting de belangrijkste oorzaak van het verloren gaan van harttransplantaten. Chronische afstoting komt niet alleen voor na harttransplantaties, maar is ook beschreven na nier-, lever-, darm- en longtransplantaties. Er bestaat momenteel geen effectieve behandeling om chronische afstoting te voorkomen waardoor een re-transplantatie in bepaalde gevallen noodzakelijk is.

De pathogenese van transplantaat arteriosclerosis is nog grotendeels onbekend, maar aannemelijk lijkt dat donor specifieke afstoting door de gastheer leidt tot het ontstaan van progressieve intima laesies.

Om het fenomeen van chronische afstoting in ons laboratorium te kunnen bestuderen introduceerden wij het allogene aorta transplantatie model in de rat. In dit model ontstaat binnen 3 weken na transplantatie transplantaat arteriosclerosis, wat het model nuttig maakt voor experimentele studies. De huidige studie had tot doel om enerzijds inzicht te krijgen in de pathogenese van transplantaat arteriosclerosis en om anderzijds de therapeutische mogelijkheden hiervan te onderzoeken.

HOOFDSTUK 2: Transplantaat arteriosclerosis in aorta transplantaten: een rol voor complement activatie en IgM antilichamen in vroege intima laesies.

Wij bestudeerden de immuno-pathogenese van chronische afstoting in het aorta transplantatie model. In 19% van de LEW→LEW en BN→BN syngene aorta transplantaten troffen we intima laesies aan. Deze intima laesies namen niet in grootte toe na 2 weken na transplantatie. In allogene aorta transplantaten werden op week 2 in 22 % van de LEW→BN en BN→LEW intima laesies gezien, toenemend tot 87% na 8 weken. Immunohistochemie van de syngene aorta transplantaten toonde zwakke aankleuring van IgM en C5b-9 deposities in de intima. In

alle allogene transplantaten met intima laesies daarentegen, werden dichte IgM en C5b-9 neerslagen gevonden, niet alleen in de intima, maar ook in de media. Vroeg na het ontstaan van intima laesies bleken deze IgM en C5b-9 neerslagen voornamelijk geïnfiltreerde macrofagen te bevatten. Deze bevinding suggereert dat het complement systeem mogelijk indirect via een effect op macrofagen van invloed is op het ontstaan van transplantaat arteriosclerosis.

Van geactiveerd complement is bekend dat het de binding van macrofagen aan de vaatwand stimuleert en de infiltratie hiervan bevordert. Geactiveerd complement stimuleert daarnaast de functie van macrofagen, met name de produktie van IL-1. IL-1 bevordert de proliferatie van vasculaire gladde spiercellen in vitro, stimuleert de adhesie van leukocyten aan de vaatwand en vergroot de expressie van het adhesie eiwit ICAM-1.

Deze studie toonde tevens inductie aan van ICAM-1 en MHC klasse II expressie op endotheelcellen van zowel syngene als allogene aorta transplantaten. Van ICAM-1 en MHC klasse II is bekend dat ze een rol spelen bij het ontstaan en het in stand houden van vasculitis. In syngene aorta transplantaten is deze vasculitis tijdelijk van aard en resulteert het mogelijk in "self-limiting" intima hyperplasie. In de allogene situatie ontstaat ten gevolge van donor specifieke afstoting een langdurige vasculitis die waarschijnlijk leidt tot progressieve transplantaat arteriosclerosis.

HOOFDSTUK 3: Immunologische gebeurtenissen vroeg na aorta transplantatie: een studie naar de effecten van ischemie.

Studies hebben aangetoond dat zowel alloantigeen afhankelijke als onafhankelijke factoren betrokken zijn bij de ontwikkeling van chronische afstoting. Het is nog onduidelijk of ook ischemie hier een rol in heeft. Sommige klinische studies laten een positieve invloed van ischemie op chronische afstoting zien, terwijl in andere studies geen effect wordt gevonden. Ook diermodellen geven geen eenduidig beeld omtrent het effect van ischemie op transplantaat arteriosclerosis. Daarom zochten we in deze studie naar een mogelijke correlatie tussen transplantaat arteriosclerosis en ischemie in het aorta transplantatie model. Om ischemie te induceren, werden de donor aorta's 1 uur lang gepreserveerd bij kamertemperatuur in fysiologisch zout alvorens ze te transplanteren in de ontvanger. Na 3 uur reperfusie werden

de ratten opgeofferd waarna de aorta transplantaten verwerkt werden voor immunohistochemie. In zowel syngene als allogene aorta transplantaten werden IgM en C5b-9 complexen aangetroffen focaal onder de endotheellaag en in de media. Deze IgM en C5b-9 neerslagen tonen overeenkomst met de neerslagen die in vroege intima laesies worden gevonden (hoofdstuk 2). Deze observatie suggereert dat ischemie de ontwikkeling van transplantaat arteriosclerosis stimuleert door activatie van het complement systeem en dat het ontstaan van transplantaat arteriosclerosis geremd kan worden door het complement systeem te blokkeren.

HOOFDSTUK 4: Transplantaat arteriosclerosis in aorta transplantaten: een rol voor cytokinen in progressieve intima verdikking.

In ons aorta transplantatie model werd een relatie tussen mRNA cytokine expressie in de vaatwand en de ernst van transplantaat arteriosclerosis aangetoond. Het cytokine mRNA profiel liet significant verhoogde mRNA expressies voor IL-1β, IL-6, IFN-γ, IL-2 en TNF-α in allogene aorta transplantaten zien. Deze verhoogde cytokine expressie vonden wij op vroege tijdstippen in de "zwakke" BN→WAG combinatie, wanneer de intima proliferatie nog progressief was. In het omgekeerde WAG→BN model met progressieve arteriosclerosis werden op alle tijdstippen na transplantatie verhoogde mRNA cytokine expressie gevonden. In syngene aorta transplantaten zagen wij alleen een verhoogde mRNA expressie voor TNF-α and TGF-β1 na respectievelijk 2 en 3 weken. Deze bevindingen tonen aan dat de expressie voor cytokine mRNA in de vaatwand verhoogd is na aorta transplantatie en dat het vrijkomen van cytokinen in de vaatwand waarschijnlijk in belangrijke mate de ernst van intima proliferatie bepaald.

HOOFDSTUK 5: Onderdrukking van acute afstoting voorkomt transplantaat arteriosclerosis na allogene aorta transplantatie in de rat.

Wij vonden een relatie tussen de ontwikkeling van transplantaat arteriosclerosis en (acute) afstoting van de vaatwand. Effectieve immunosuppressie door cyclosporine (CsA) en rapamycine voorkomt het ontstaan van intima verdikking. Na hoge CsA dosering werd zelfs minder transplantaat arteriosclerosis gevonden in BN→WAG aorta transplantaten dan in

syngene WAG aorta transplantaten die niet met CsA werden behandeld. Deze bevinding suggereert dat effectieve immunosuppressie met CsA niet alleen arteriosclerosis ten gevolge van afstoting voorkomt, maar ook arteriosclerosis als reactie op operatieve manipulatie. Lage CsA dosering gaf alleen in het BN→WAG model een significante afname te zien in de ontwikkeling van intima laesies. In de WAG→BN combinatie leidde behandeling met lage dosering CsA niet tot een significante inhibitie.

Samenvattend kwamen wij tot de hypothese dat chronische afstoting in de kliniek het gevolg zou kunnen zijn van onvoldoende suppressie van acute afstoting. Deze hypothese wordt ondersteund door diverse klinische studies die een correlatie aantonen tussen het optreden van transplantaat arteriosclerosis en het optreden van acute afstoting. Klinische studies met hoge doseringen immunosuppressiva zijn echter helaas niet mogelijk door schadelijke bijwerkingen van de diverse middelen zoals bijvoorbeeld de nefro-toxiciteit van CsA.

HOOFDSTUK 6: Ketanserine remt het ontstaan van transplantaat arteriosclerosis na allogene aorta transplantatie in de rat.

In de literatuur wordt verondersteld dat de serotonine-2 receptor antagonist ketanserine de ontwikkeling van atherosclerosis remt door zijn effect op trombocyten en op vasculaire gladde spiercellen. Wij onderzochten het effect van ketanserine op de ontwikkeling van transplantaat arteriosclerosis in het allogene rat aorta transplantatie model. Ketanserine reduceerde in een dosering van 10 mg/kg/dag alleen significant de intima proliferatie in de BN→WAG combinatie. Om het mechanisme te onderzoeken hoe ketanserine intima hyperplasie vermindert, bestudeerden wij het effect van ketanserine op collageen geïnduceerde trombocyten aggregatie. Onbehandelde BN trombocyten waren meer gevoelig voor collageen dan WAG trombocyten. Na ketanserine behandeling was de BN trombocyten-aggregatie verminderd. De vermindering van BN trombocyten-aggregatie ging echter niet gepaard met vermindering van transplantaat arteriosclerosis in het WAG→BN aorta transplantatie model. Anderzijds vonden wij een significante verminderde intima verdikking in het BN→WAG model, terwijl geen effect van ketanserine op WAG trombocyten kon worden vastgesteld. Daarom werd in deze studie geen correlatie gevonden tussen het effect van ketanserine op de

functie van trombocyten en reductie van intima verdikking. Dit suggereert dat ketanserine arteriosclerosis reduceert door een ander mechanisme dan door het verminderen van de gevoeligheid van trombocyten voor aggregatie. Een direct blokkerend effect van ketanserine op serotonine geïnduceerde proliferatie van vasculaire gladde spiercellen is een aannemelijke verklaring voor de anti-arteriosclerotische werking. In vitro is serotonine in staat de proliferatie van vasculaire gladde spiercellen te stimuleren. De slechts matige inhibitie van transplantaat arteriosclerosis door ketanserine in deze studie impliceert een kleine rol voor serotonine in het gecompliceerde mechanisme leidend tot de ontwikkeling van transplantaat arteriosclerosis.

ABBREVIATIONS

ACEI:	angiotensin converting enzyme inhibitor
BN:	Brown Norway
C5b-9:	complement membrane attack complex
CMV:	cytomegalovirus
CsA:	cyclosporine A
g:	gram
GVD:	graft vascular disease
h:	hour
HLA:	human leucocyte antigen
ICAM:	intercellular adhesion molecule
IFN:	interferon
IL:	interleukin
LPS:	lipopolysaccharide
LEW:	Lewis
μm:	micrometer
MHC:	major histocompatibility complex
mRNA:	messenger ribonuclease
n:	number of observations/animals
NGS:	normal goat serum
p:	level of significance
PCR:	polymerase chain reaction
PDGF:	platelet derived growth factor
RAPA:	rapamycin
TBS:	tris buffered saline
TGF:	transforming growth factor
TNF:	tumor necrosis factor
VSMC:	vascular smooth muscle cell

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Rob Anton Geerling was born in Tietjerk (Friesland, the Netherlands) on 5 december 1969. He visited grammar school in Arnhem (Stedelijk Gymnasium) from 1982 to 1985 and graduated in 1988 at the Theresia Lyseum in Tilburg. In 1988 he started Medical school in Rotterdam (Erasmus University). During his medical study he was student assistent at the Laboratory for Experimental Surgery under chief of Prof.dr. J. Jeekel. In 1992 he started the study on chronic rejection in the aortic transplantation model in the rat. He continued this study in laboratories of the Department of Immunology and Infectious Diseases and Cardiovascular Surgery at the Johns Hopkins School of Medicine, Baltimore, USA (head: Prof. A. Herskowitz). After his Medical degree in april 1996, he started his clinical training for ophtalmologist at the department of ophtalmology, Academic Hospital Rotterdam, during which many of the writings still had to be done.

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