Transpl Int (2004) 17: 9–14
DOI 10.1007/s00147-003-0653-8

ORIGINAL ARTICLE

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Differential effects of modern immunosuppressive agents on the development of intimal hyperplasia

Received: 30 July 2002
Revised: 3 June 2003
Accepted: 8 July 2003
Published online: 10 October 2003

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Abstract Modern immunosuppressive agents such as tacrolimus and rapamycin are claimed to be associated with a reduction in vascular narrowing, a central feature of chronic rejection. This study assesses the effect of cyclosporine, tacrolimus and rapamycin on the development of intimal thickening, fibrosis-associated genes and deposition of extracellular matrix (ECM) proteins in a model of intimal hyperplasia. Male Sprague-Dawley rats received either no treatment or 5 mg/kg cyclosporine, 0.1 mg/kg tacrolimus or 0.05 mg/kg rapamycin. Animals underwent left common carotid balloon angioplasty, and intima medial ratios, pro-fibrotic gene expression and ECM accumulation were calculated at 14 and 28 days. Cyclosporine was associated with increased intimal thickening compared to controls ($P < 0.004$). Tacrolimus had no effect on intimal thickening, whilst rapamycin significantly inhibited intimal thickening at both 14 and 28 days ($P < 0.004$ and $P < 0.026$, respectively). All groups significantly inhibited matrix metalloproteinase (MMP)-2, MMP-9, tissue inhibitor of metalloproteinases (TIMP)-1, transforming growth factor (TGF)-β and collagen III expression at 14 days ($P < 0.001$), but increased ECM deposition. However, rapamycin marginally reduced ECM deposition compared to cyclosporine ($P < 0.06$). Treatment with cyclosporine was associated with worsening of vascular narrowing, whilst rapamycin showed a beneficial reduction in intimal thickening. Treatment with all immunosuppressive agents resulted in increased ECM deposition. Rapamycin may halt the progression of vascular narrowing compared to both cyclosporine and tacrolimus.

Keywords Intimal hyperplasia · Metalloproteinase · Extracellular matrix

Introduction

Vascular narrowing occurring in transplanted organs, also termed allograft vasculopathy, is a central feature of chronic rejection in all solid organ allografts [1]. Allograft vasculopathy is characterised histologically by intimal hyperplasia that affects both arteries and veins, producing luminal encroachment, end-organ ischaemia and eventual graft failure. Intimal hyperplasia results from vascular smooth muscle cell proliferation, migration and deposition of extracellular matrix (ECM) proteins. Pro-fibrotic cytokines, matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) are pivotal mediators in this process. The mechanisms underlying this process are incompletely understood, but it is thought that a number of

immunological and non-immunological aetiological factors are involved, including the effects of immunosuppressive regimens, not only on acute rejection episodes, but also independently on the development of chronic rejection.

Despite improvements in early allograft survival since the introduction of cyclosporine in the 1980s [2], long-term graft survival remains unchanged [3]. Cyclosporine has no effect on chronic rejection and in fact has been shown to accelerate allograft vasculopathy [4, 5] and fibrosis [6]. Tacrolimus (FK506) is a macrolide antibiotic with potent immunosuppressive properties associated with a reduction in acute rejection episodes [7, 8]. Tacrolimus has a similar mechanism of action to cyclosporine and also inhibits calcineurin-phosphatase. Its role in the development of allograft vasculopathy still remains to be defined [9]. Currently there is much interest in a novel immunosuppressive and anti-proliferative drug called rapamycin. Experimental evidence suggests a potential role for rapamycin in both the prevention and treatment of allograft vasculopathy [10, 11]. Immunosuppressive agents that may prevent the development or progression of allograft vasculopathy therefore offer promising prospects for prolonging long-term graft survival. The aim of this study was to assess the role of these immunosuppressive agents on the development of intimal thickening, genes associated with fibrosis and ECM matrix deposition in a model of intimal hyperplasia. This non-allogenic model was chosen in order to assess the effects of these drugs independently of their immunosuppressive properties.

Materials and methods

Arterial injury model

Four-month-old male Sprague-Dawley rats (weighing 350–400 g) were obtained from Harland (Cambridge, UK). Rats were assigned to receive no treatment, cyclosporine (5 mg/kg per day), tacrolimus (0.1 mg/kg per day) or rapamycin (0.05 mg/kg per day) by oral gavage 3 days pre-operatively and until the end of the study. Six rats were used in each treatment group for each time point assessed. All rats underwent left common carotid balloon angioplasty as described previously [12]. In brief, a 2F balloon embolectomy catheter (Baxter Healthcare) was introduced through the left external carotid artery into the common carotid artery. The catheter was inflated to a standard pressure of 2 atm and passed in a twisting manner from the arch of the aorta to the carotid bifurcation, denuding the common carotid artery of its endothelium. This procedure was repeated twice more before the catheter was removed and the external carotid ligated. Rats were anaesthetised at 14 and 28 days after balloon injury and the left common carotid artery was flushed with phosphate-buffered saline. The middle third of each vessel was used for histomorphometry and the outer thirds were snap-frozen in liquid nitrogen and stored at −70°C for molecular studies.

Morphology

Vessels for histological study were fixed with 2.5% gluteraldehyde in 0.1 M phosphate buffer (pH 7.4). After 16 h of further fixation, the vessels were embedded in epoxy resin and cut into 4-μm transverse sections. Intima and medial areas were assessed using light microscope-assisted computer planimetry. Intima and medial areas were defined by internal and external elastic laminae, respectively.

Molecular analysis

The methods used to quantify levels of messenger RNA expression using reverse transcriptase-polymerase chain reaction (RT-PCR) have been described previously [13]. Vessels were cut into 4-μm sections and mRNA was extracted using oligo-dT-linked Dynabeads (Dynal, Bromborough, UK). Competitive RT-PCR was performed to quantify genes of interest including matrix metalloproteinases (MMPs)-2 and -9, tissue inhibitor of metalloproteinases (TIMP-1), matrix protein collagen III and transforming growth factor-β (TGF-β). All probes and primers were designed from sequences available on the EMBL database using the program GCG prime (Genetics Computer Group, Madison, Wisc.) and synthesised by Life Technologies (Paisley, UK) or Genosys Biotechnologies Europe (Pampisford, UK). Quantification of RT-PCR products was performed using an enzyme-linked immunosorbent assay. Differences in tissue cellularity were corrected for by expressing values of complementary DNA product as a ratio to that of constitutively expressed housekeeping gene, β-actin.

Sirius red staining

Carotid arteries were formalin-fixed, embedded in epoxy resin and cut into 4-μm sections. The resin was removed in xylene for 10 min and dehydrated in serial washes of 100% alcohol for 2 min performed twice and 95%, 80% and 60% alcohol for 2 min. Sections were run under cold running water for 10 min and finally rinsed briefly with distilled water. Staining was performed overnight for 12 h in picro-sirius red F3BA. Rapid dehydration was repeated with an initial wash of 0.01 M HCl for 2 min and serial washes of alcohol. Slides were cleared with two washes of xylene for 2 min, excess xylene was removed and slides were mounted with Xam organic mountant. Ten random windows of intima were analysed and the value of ECM was determined from the mean of these windows.

Results

Histology

There were significant differences between all groups in the development of intimal hyperplasia at 14 and 28 days (Kruskal-Wallis: $P < 0.001$) (Figs. 1 and 2). Cyclosporine had no effect on the development of intimal hyperplasia at 14 days compared to untreated controls ($P < 0.53$). Importantly, its long-term administration was associated with an increase in intimal thickening compared to untreated controls ($P < 0.004$). Tacrolimus significantly inhibited intimal hyperplasia at

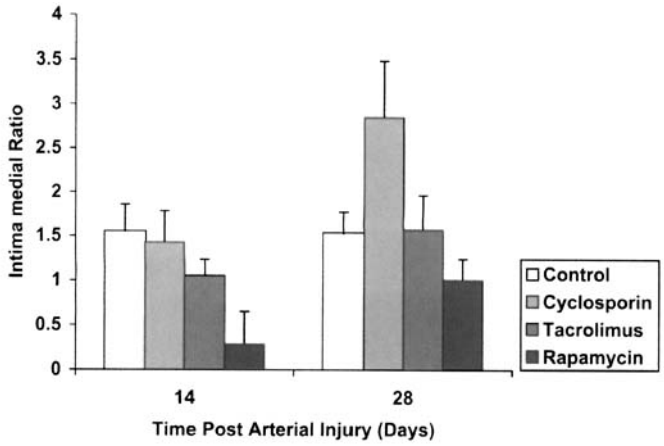

Fig. 1 Bar graph showing intima medial ratios at 14 and 28 days after arterial balloon injury. Intima medial ratios were significantly reduced in both the tacrolimus and rapamycin groups at 14 days, but only in rapamycin-treated animals at 28 days. Cyclosporine was associated with a significant increase in intimal thickening compared to untreated controls at 28 days

14 days compared to controls ($P < 0.014$), but this was not significant at 28 days. Treatment with rapamycin significantly inhibited intimal thickening compared to controls, cyclosporine- and tacrolimus-treated animals at 14 days ($P < 0.004$ for all groups) and at 28 days ($P < 0.026$ for all groups).

Pro-fibrotic gene expression

Dosing of animals with cyclosporine, tacrolimus and rapamycin had no effect on the expression of the housekeeping gene β-actin compared to untreated controls (data not shown). All treatment groups significantly inhibited the expression of MMP-2, MMP-9, TIMP-1, TGF-β and collagen III at 14 days compared to untreated controls (Kruskal-Wallis: $P < 0.001$ for all genes) (Figs. 3, 4, 5, 6). There were no differences in pro-fibrotic gene expression between treatment groups. However, when metalloproteinases were expressed as a ratio to TIMP-1 expression, MMP-2:TIMP-1 and MMP-9:TIMP-1 were significantly higher in animals receiving cyclosporine ($P < 0.01$) compared to untreated controls and treatment with either tacrolimus or rapamycin.

Extracellular matrix deposition

All treatment groups were associated with a significant increase in intimal ECM deposition (Kruskal-Wallis: $P < 0.001$) (Fig. 7). Cyclosporine was associated with the greatest increase compared to untreated controls ($P < 0.004$). There was no difference in ECM deposition between the cyclosporine and tacrolimus groups, but treatment with rapamycin showed a marginally significant reduction of ECM compared to cyclosporine ($P < 0.06$).

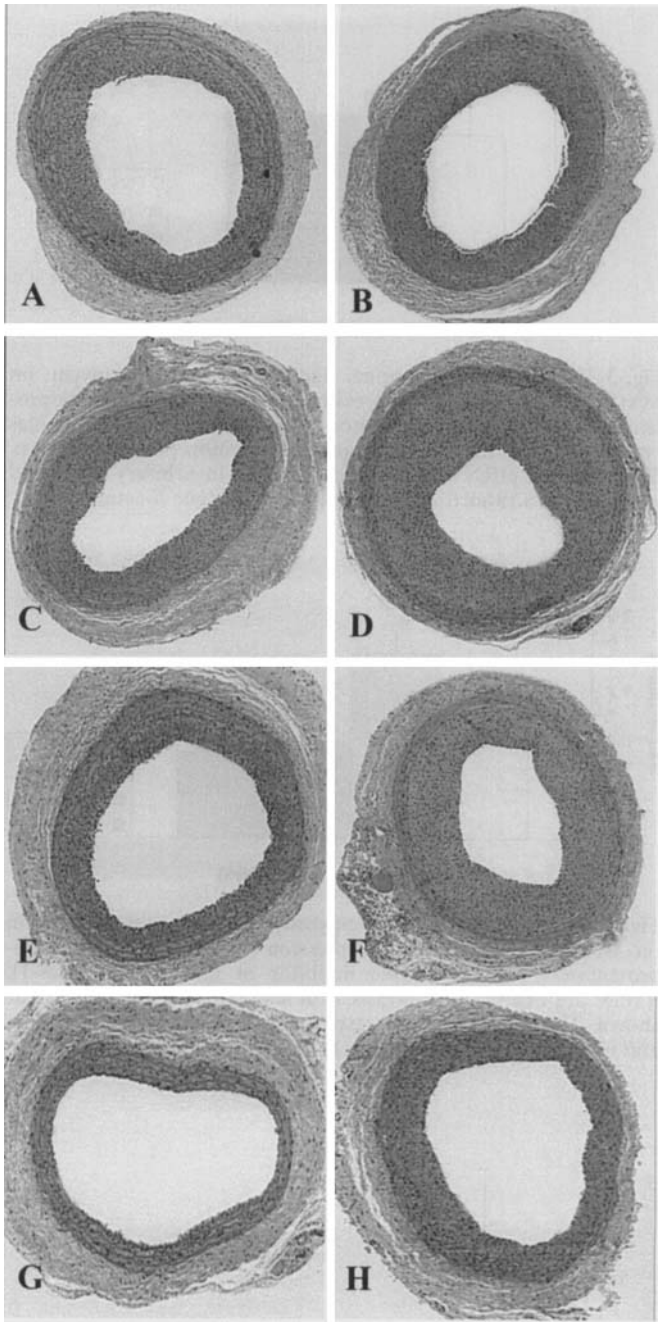

Fig. 2 Representative transverse cross-sections of carotid artery demonstrating the effect of cyclosporine (5 mg/kg per day), tacrolimus (0.1 mg/kg per day) and rapamycin (0.05 mg/kg per day) on intimal hyperplasia. Control vessels (**A** and **B**), cyclosporine (**C** and **D**), tacrolimus(**E** and **F**) and rapamycin (**G** and **H**) are shown 14 and 28 days after balloon injury, respectively

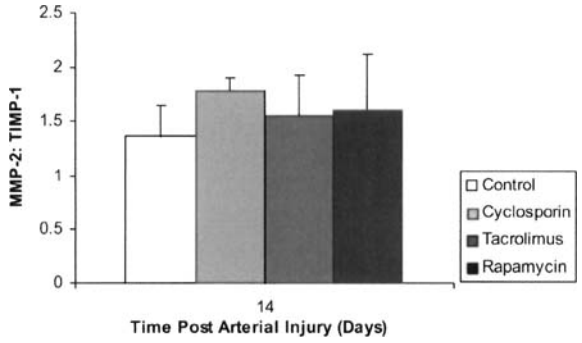

Fig. 3 Effects of cyclosporine, tacrolimus and rapamycin on MMP-2:TIMP-1 mRNA expression (*MMP-2* matrix metalloproteinase-2, *TIMP-1* tissue inhibitor of metalloproteinases-1). Values are expressed as means with standard deviation of means shown. The value of mRNA expression is stated in arbitary units and expressed as a ratio to that of housekeeping gene β-actin

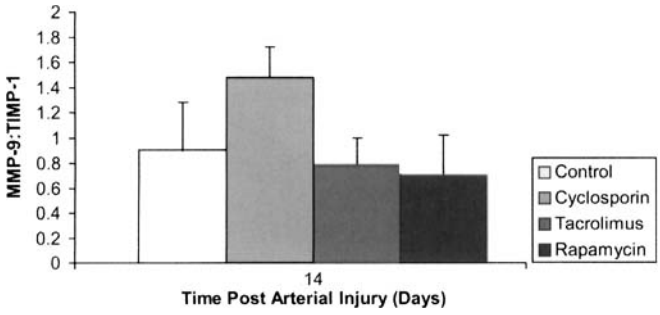

Fig. 4 Temporal effects of cyclosporine, tacrolimus and rapamycin on MMP-9:TIMP-1 mRNA expression (*MMP-9* matrix metalloproteinase-9, *TIMP-1* tissue inhibitor of metalloproteinases-1). Values are expressed as means with standard deviation of means shown. The value of mRNA expression is stated in arbitary units and expressed as a ratio to that of housekeeping gene β-actin

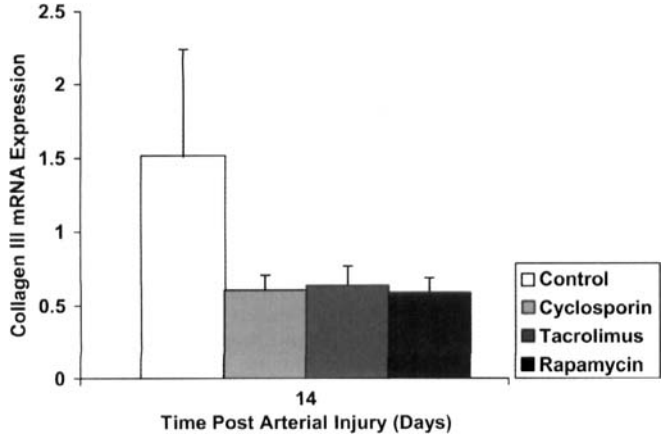

Fig. 5 Effects of cyclosporine, tacrolimus and rapamycin on collagen III mRNA expression. Values are expressed as means with standard deviation of means shown. The value of mRNA expression is stated in arbitary units and expressed as a ratio to that of housekeeping gene β-actin

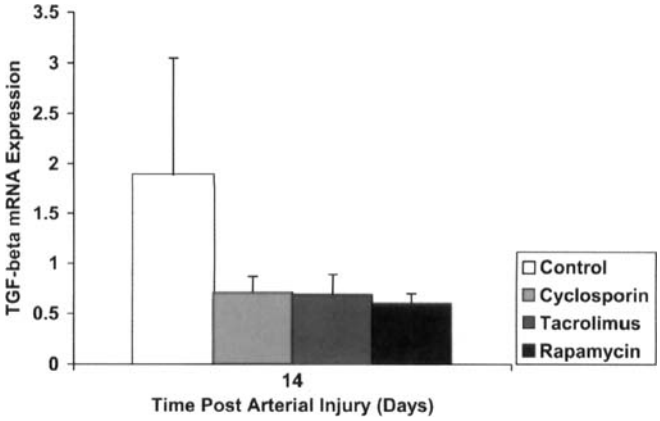

Fig. 6 Effects of cyclosporine, tacrolimus and rapamycin on transforming growth factor (*TGF*)-β mRNA expression. Values are expressed as means with standard deviation of means shown. The value of mRNA expression is stated in arbitary units and expressed as a ratio to that of housekeeping gene β-actin

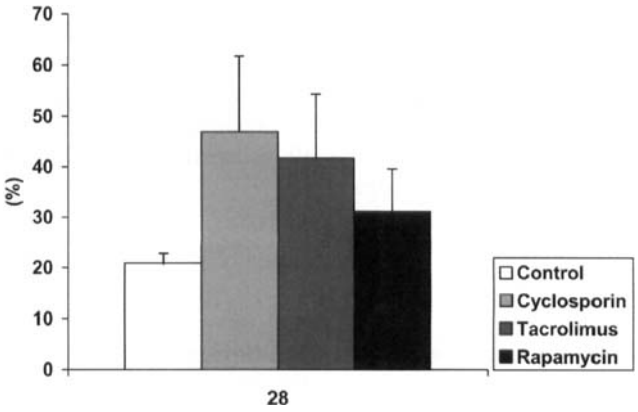

Fig. 7 Graph showing the effect of cyclosporine, tacrolimus and rapamycin on intimal extracellular matrix deposition 28 days after balloon angioplasty

Discussion

This study uses a mechanical model of arterial injury to study the effects of modern immunosuppressive agents on the development of intimal hyperplasia, the histological hallmark of allograft vasculopathy. Whilst the mechanisms leading to intimal hyperplasia in both mechanical and immune-mediated models result from different responses to arterial injury, the central processes leading to intimal thickening are comparable and mediated by similar growth factors and proteases [14, 15, 16]. In this study, immunosuppression doses comparable to those used in human organ transplantation were chosen to reflect clinical practice.

The findings of this study show cyclosporine to have no inhibitory effect on vascular smooth muscle cell migration or deposition of ECM proteins. Importantly, prolonged treatment with cyclosporine to 28 days after arterial injury is associated with significantly increased intimal thickening and deposition of ECM proteins compared to controls. Tacrolimus has a significant inhibitory effect on early intimal hyperplasia, but no effect on late intimal thickening. However, this is significantly less than cyclosporine. The lack of inhibitory effect of cyclosporine and tacrolimus is not surprising, as both agents are known to have little effect beyond blocking transcription of cytokine-dependent T-cell proliferation [17]. However, we have shown rapamycin to have a significant inhibitory effect on intimal thickening at both 14 and 28 days. This finding has been previously demonstrated in other models of smooth muscle cell proliferation [18], but rapamycin was administered at 1.5 mg/kg per day, 30 times the dose used in this study and the clinical setting. Animals treated with rapamycin showed a significant increase in intimal thickening at 28 days compared to 14 days, suggesting its mechanism of action is not due to a cytotoxic effect on vascular smooth muscle cells.

Rapamycin is known to have anti-proliferative properties distinct from its immunosuppressive ones. Unlike cyclosporine and tacrolimus, rapamycin does not inhibit calcineurin-phosphatase but, like tacrolimus, binds to an intracellular cyclophyllin known as FK12-BP. However, this complex does not inhibit calcineurin-phosphatase, but binds to mammalian targets of rapamycin (mTOR) arresting growth-factor-mediated cell cycle progression from G_1 to DNA synthesis phase [19]. This inhibitory effect is not limited to T cells, but also inhibits fibroblasts, endothelial and vascular smooth muscle cells as well as lymphocytes, all pivotal cells in the development of allograft vasculopathy [20]. Rapamycin has proved a potent immunosuppressive agent in the clinical setting [21, 22], but its role in the development of allograft vasculopathy remains to be defined. Recently, Ikonen et al. have shown rapamycin to both inhibit the progression of pre-vasculopathy and regress established allograft vasculopathy in cynomolgus monkeys [23], suggesting promising prospects for clinical practice.

The absolute values for all three agents on pro-fibrotic gene expression are comparable in this study and fail to explain the subsequent differences in intimal thickening. However, it is the relative balance between metalloproteinases and their inhibitors that is important. Significant differences in the ratios between MMP-2 and MMP-9:TIMP-1 mRNA expression have clearly been shown in this model compared to untreated controls. Cyclosporine resulted in a significant increase in both MMP-2:TIMP-1 and MMP-9:TIMP-1 expression compared to untreated controls. Furthermore, MMP-9:TIMP-1 was significantly elevated in cyclosporine animals compared to treatment with either tacrolimus or rapamycin and may account for increased intimal thickening in cyclosporine-treated animals compared to controls and other treatments. Vascular smooth muscle cells (VSMCs) are not normally resident in the uninjured rat intima [24], and their presence is the result of VSMC proliferation and migration through the internal elastic lamina. For a cell to migrate, it must free itself from its surrounding extracellular architecture. Expression and production of metalloproteinases facilitate this process [25], whilst a relative increase in the production of TIMPs is inhibitory [26]. TIMPs form a 1:1 stoichiometric complex with MMPs inhibiting their activity.

Collagen III is the major constituent of the arterial wall and comprises 20–50% of the dry weight [27]. Interestingly, all treatment groups were associated with increased ECM deposition compared to untreated controls. This was observed in animals in the rapamycin group, despite an overall significant reduction in intimal thickening at both early and late end points. TGF-β and collagen III expression failed to explain this difference or the trend to statistical significance ($P < 0.06$) between cyclosporine and rapamycin. However, ECM accumulation was measured using sirius red staining. This stain is not selective for collagen III, but stains for all the components of the ECM. Furthermore, the accumulation of ECM is complex and whilst ECM is increased in all treatment groups, despite decreased transcription of collagen III mRNA at 14 days, this study does not address post-translational modification known to occur at both intracellular and extracellular sites [28]. Presently there are no data in the literature to explain the observed differences in ECM deposition.

In summary, the findings of this study suggest that cyclosporine, tacrolimus and rapamycin have significantly different effects on the development of vascular narrowing, independent of their immunosuppressive properties. This study highlights the important finding that all three drugs are associated with an increase in ECM deposition compared to untreated controls. The accumulation of ECM proteins, or fibrosis, is a fundamental process of allograft damage leading to the failure of the transplanted organ. Rapamycin compares favourably with both cyclosporine and tacrolimus in terms of vascular narrowing and ECM deposition, and results are eagerly awaited to assess its role in the prevention of allograft vasculopathy to prolong organ allograft survival.

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