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ORIGINAL ARTICLE

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The effect of combined rapamycin/cyclosporine on the changes in pro-fibrotic gene expression that occur during the development of allograft vasculopathy in rats, compared with cyclosporine or rapamycin in isolation

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Abstract Chronic allograft dysfunction, the leading cause of solid-organ transplant failure, is characterised by histological evidence of extracellular matrix (ECM) accumulation (fibrosis). The aim of this study was to compare the effect of combined rapamycin and cyclosporine therapy on fibrosis-associated gene expression and ECM turnover during the development of allograft vasculopathy, compared with either agent alone. Lewis recipients of F344 rat thoracic-to-abdominal aorta transplants were administered rapamycin, cyclosporine, combined rapamycin and cyclosporine or no treatment. F344-to-F344 isografts served as controls. Six grafts in each group were harvested at 16 weeks. Vascular remodelling and ECM accumulation (Sirius red) were measured by computerised histomorphometry of aortic sections. Messenger RNA was extracted from frozen tissue, and expression of fibrosis-associated genes was studied by means of semi-quantitative reverse transcription polymerase chain reaction (RT-PCR). Rapamycin (0.5 mg/kg per day) or cyclosporine (5 mg/kg per day) inhibited intimal hyperplasia, medial ECM accumulation and expansive vascular remodelling (increasing vessel circumference) in rat aortic allografts. This was associated with attenuation of the graft inflammatory infiltrate and a reduction in intra-graft gelatinase, collagen III and tissue inhibitor of metalloproteinase (TIMP)-1 mRNA levels. Combined rapamycin and cyclosporine inhibited intimal hyperplasia; however, there was a lesser effect on vascular remodelling and medial ECM accumulation. Combined-treatment aortic allografts were also seen to have a more-severe inflammatory infiltrate and larger amounts of intra-graft matrix metalloproteinase (MMP)-9, transforming growth factor (TGF)-β and TIMP-1 mRNA than those treated with monotherapy. Rapamycin and cyclosporine act synergistically to inhibit intimal hyperplasia but not the inflammatory infiltrate, allograft fibrosis or vessel remodelling. In the high-responder F344-to-Lewis rat model, effective immunosuppression is required to reduce graft fibrosis.

Keywords Cyclosporine · Rapamycin · Vascular remodelling · Allograft vasculopathy · Metalloproteinases Fibrosis

Introduction

The development of chronic allograft dysfunction (CAD) is the most important clinical problem in solid-organ transplantation, accounting for the majority of graft losses after the first year post-transplantation [7]. CAD occurs as a consequence of the fibroproliferative response to ongoing graft injury and inflammation and is characterised histologically by the accumulation of extracellular matrix (ECM; fibrosis) and vascular remodelling (allograft vasculopathy). The effectiveness of cyclosporine (CyA) at preventing or slowing the progression of CAD is limited by its toxicity profile as well as variable bioavailability that can lead to impaired efficacy. Attempts to address these problems by the use of combinations of CyA with a second immunosuppressive agent have not, however, been shown to reduce allograft fibrosis or vasculopathy significantly. Rapamycin (Rapa), a macrolide antibiotic that has been shown to inhibit the fibroproliferative tissue response in rodents [5], has recently entered clinical practice in combination with CyA. It has been shown to prevent allograft dysfunction in a wide range of animal models [15], and in rats, in vivo, CyA and Rapa act in synergy to attenuate cell-mediated rejection in high-responder combinations [17]. The effect of these two agents in combination on the progression of CAD remains to de determined, however. The aim of this study was to compare the effect on fibrosis-associated gene expression, ECM accumulation and vascular remodelling that underlie the fibroproliferative response in rat allografts, of combined Rapa and CyA therapy or either agent in isolation.

Materials and methods

Animal model

The thoracic-aorta to abdominal-aorta allograft model as described by Mennander et al. [9] was used. A segment of the descending thoracic aorta, approximately 3 cm in length, was excised, thoroughly perfused with phosphate-buffered saline (PBS) and used as a transplant. F344 rats acted as donors and recipients, and Lewis rats as recipients. Ischaemic injury to the graft was minimised by immersion of the graft in an ice bath at 4 °C between procedures. Through a midline laparotomy incision the segment of thoracic aorta was anastomosed end to end to the recipient abdominal aorta by use of 9/0 Prolene suture. The graft was transplanted into heterotopic position below renal arteries and above the bifurcation forming a loop in the recipient abdomen.

Animals were kept in a controlled environment with unlimited access to feed and water. Graft harvest was performed in anaesthetised rats, following which the animal was allowed to die by exsanguination. Experiments were performed under UK Home Office licence PPL 80/1434, The Animals (Scientific Procedures) Act 1986.

Drugs

Rapamycin (sirolimus; Wyeth-Ayerst, Princeton, N.J., USA) was prepared twice weekly as two solutions of 5 and 0.5 mg/ml in distilled water for experimental groups 2 and 5, respectively. Solutions were stored at 4 °C and protected from UV light exposure. Neoral cyclosporine (Novartis, UK) was prepared as two solutions of 5 and 1.5 mg/ml in olive oil and stored at 21 °C. Drugs were administered orally by gavage from the day of transplantation until the animals were killed. The drug doses of Rapa and CyA were chosen because of their efficacy at inhibiting allograft vasculopathy in this model, their known pharmacokinetics and the absence of toxicity at these doses. The combined CyA and Rapa doses were determined as a ×3 reduction in the CyA dose and a ×10 reduction in the Rapa dose. This level of dose reduction has previously been shown, using median effect analysis, to be equally efficacious at prolonging allograft survival in Lewis rats, compared with either drug in isolation [15].

Experimental groups

- Group 1: F344-to-F344 isografts (isografts, negative control)
- Group 2: F344-to-Lewis allografts receiving CyA at 5 mg/kg per day orally by gavage for 14 days only, then no immunosuppression until killing (untreated allografts, positive control)
- Group 3: F344-to-Lewis allografts receiving CyA at 5 mg/kg per day (cyclosporine)
- Group 4: F344-to-Lewis allografts receiving Rapa at 0.5 mg/kg per day (rapamycin)
- Group 5: F344-to-Lewis allografts receiving combined CyA at 1.5 mg/kg per day and Rapa at 0.05 mg/kg per day (Rapa + CyA)

Six rats in each group were killed at 16 weeks.

Allograft gene expression using RT-PCR

Total mRNA was extracted from aortic tissue, and complementary DNA molecules were synthesised by reverse transcription (RT). These cDNA species were amplified by polymerase chain reaction (PCR) and quantified in an ELISA system. Relative quantitation was performed by comparison of the signal intensity with that of the housekeeping gene β-actin. These techniques have been described in more detail elsewhere [2]. Probe and primer sequences used in these experiments have also been published previously [6].

Histological analysis

Segments of the aortic graft were embedded in paraffin wax, and multiple sections were stained with haematoxylin and eosin. The level of inflammatory infiltrate was assessed semi-quantitatively and was categorised as follows: none, no inflammatory cells visualised; mild, endotheliitis, inflammatory cells localised to the endothelium; moderate, dense inflammatory cell infiltrate in the innermost layer of the vessel wall; severe, dense inflammatory cell infiltrate throughout all layers of the vessel wall.

The vessel circumference and area fraction of the intima and media were quantified by a computer image-analysis system. The intima was defined as the area between the endothelium and the internal elastic lamina, and the media was defined as the area between the internal and external elastic laminas. Expansive remodelling was defined as an increase in vessel (internal elastic lamina) circumference. Paraffin-embedded sections were also stained with Sirius-red stain, and the level of ECM staining was quantified by computerised histomorphometry as described previously [10].

Substances to be assessed

In order to take into account all the factors which influence smooth muscle cell proliferation, migration and deposition of ECM during intima formation, we studied the following species with RT-PCR: matrix metalloproteinase (MMP)-9, tissue inhibitor of metalloproteinase (TIMP)-1, 2 and 3, transforming growth factor (TGF)-β and collagen III.

Analysis of results

The levels of gene expression were compared in the different study groups by use of non-parametric statistical analysis. Mann–Whitney comparisons were made between individual groups only where Kruskal–Wallis analysis suggested significance. Categorical values were compared with Pearson's χ^2-test. Statistical analysis was performed with the Statistical Package for the Social Sciences Version 8.0 (Chertsey, UK).

Results

Results of histology

Untreated allografts developed significant intimal thickening at 16 weeks compared with isografts and also underwent progressive expansive remodelling (Figs. 1 and 2). CyA significantly inhibited both intimal hyperplasia (intima media ratio 0.08 ± 0.09 vs 0.2 ± 0.05, $P = 0.01$, Mann–Whitney, Fig. 2a) and expansive vascular remodelling (internal elastic lamina circumference $3{,}845 \pm 196$ vs $4{,}203 \pm 175$ μm, $P = 0.02$, Fig. 2b) when compared with untreated allografts, as well as significantly attenuating the severity of the inflammatory infiltrate ($P < 0.001$, Pearson's χ^2-test, Yates' correction, Fig. 2c). Rapa also inhibited intimal hyperplasia (intima media ratio 0.01 ± 0.1, $P = 0.08$, Mann–Whitney), expansive remodelling (internal elastic lamina circumference $3{,}950 \pm 150$ μm, $P = 0.016$, Mann–Whitney) and inflammation, when compared with untreated allografts. Combination therapy inhibited intimal hyperplasia (intima media ratio 0.01 ± 0.1, $P = 0.08$, Mann–Whitney), but failed to inhibit expansive remodelling (internal elastic lamina circumference $3{,}950 \pm 150$ μm, $P = 0.016$, Mann–Whitney) or the allograft inflammatory infiltrate (Fig. 2c).

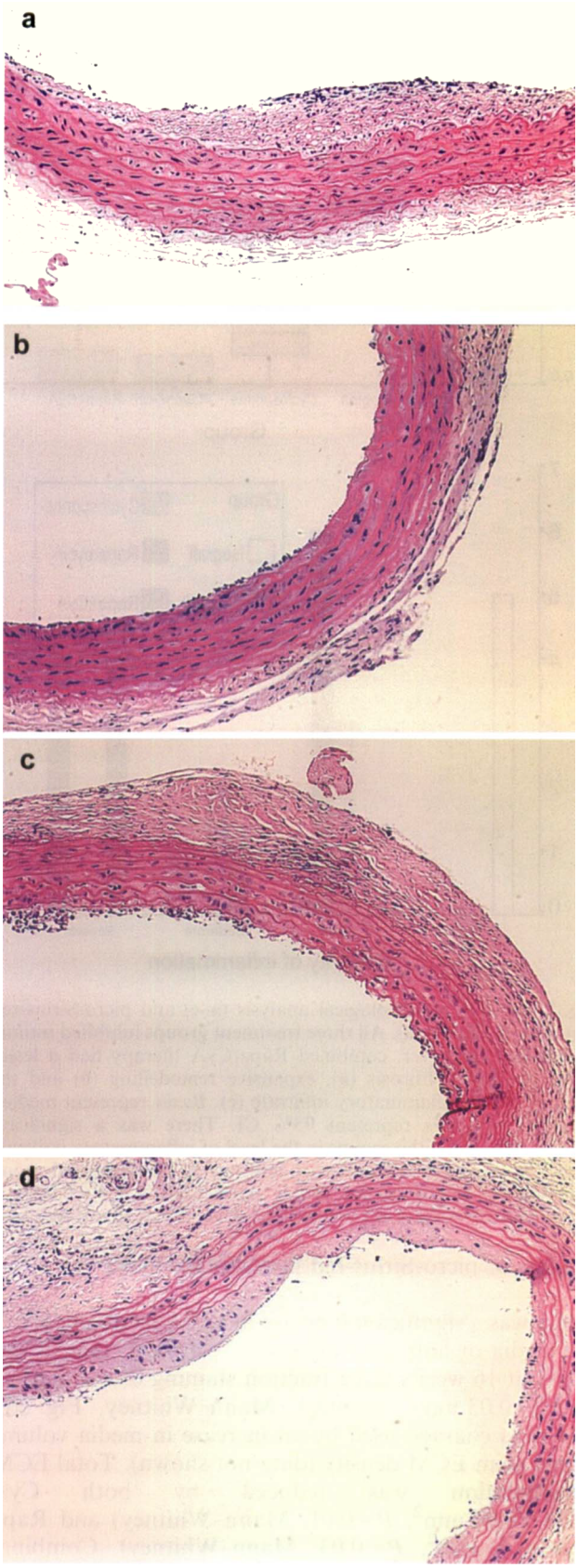

Fig. 1a–d Representative photomicrographs of haematoxylin & eosin-stained sections of rat aortas at 16 weeks. **a–c** Allografts treated with **a** cyclosporine (5 mg/kg per day, weeks 0–16), **b** rapamycin (0.5 mg/kg per day, weeks 0–16), and **c** combined rapamycin (0.05 mg/kg per day, weeks 0–16) and cyclosporine (1.5 mg/kg per day, weeks 0–16). **d** Allograft treated with cyclosporine (5 mg/kg per day, weeks 0–2) as positive control. Positive-control allografts have a dense transmural inflammatory infiltrate associated with intimal, medial and neo-adventitial thickening. These changes were attenuated by Rapa and CyA in isolation; however, combined Rapa/CyA failed to inhibit the inflammatory infiltrate, medial fibrosis or neo-adventitia formation in allografts

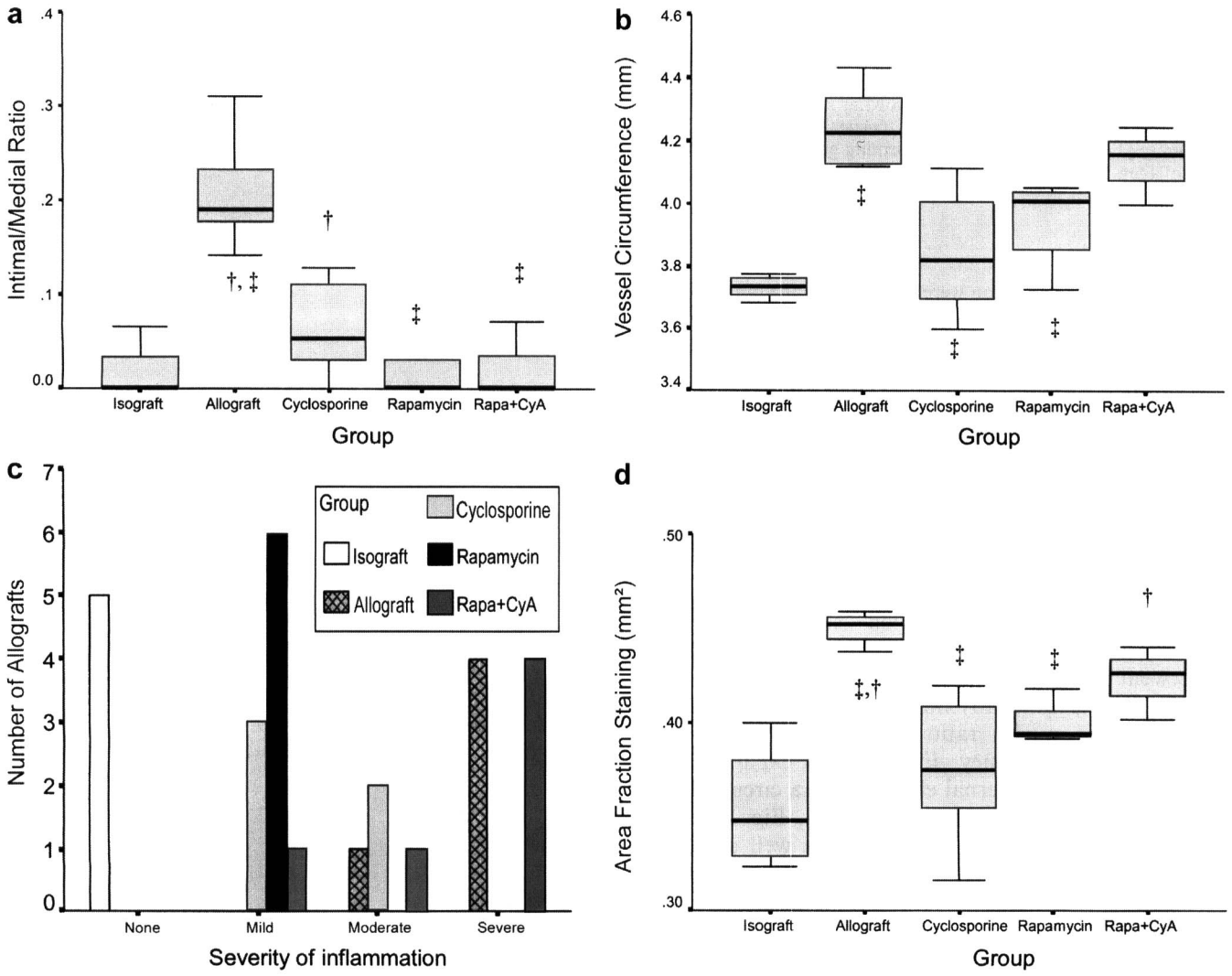

Fig. 2 Results of histological analysis (**a–c**) and picro-Sirius-red histomorphometry (**d**). All three treatment groups inhibited intimal hyperplasia; however, combined Rapa/CyA therapy had a lesser effect on medial fibrosis (**a**), expansive remodelling (**b**) and the severity of the inflammatory infiltrate (**c**). *Boxes* represent median plus SD, *whiskers* represent 95% CI. There was a significant difference between the groups in the level of inflammatory infiltrate with a more severe inflammatory infiltrate in the combined group (Pearson's χ^2-test, $P<0.001$, Yates' correction). **a** ‡ $P=0.0001$, † $P=0.01$; **b** ‡ $P=0.001$; **d** ‡ $P=0.001$, † $P=0.08$, all Mann–Whitney

Results of picro-Sirius-red histomorphometry

There was a significant increase in ECM accumulation in the media of untreated allografts relative to that of isografts at 16 weeks (area fraction staining $0.45\pm$ 0.01 vs $0.35\pm$ 0.03 mm^2, $P=0.03$, Mann–Whitney, Fig. 2d). This was characterised by an increase in media volume rather than ECM density (data not shown). Total ECM accumulation was reduced by both CyA (0.38 ± 0.04 mm^2, $P=0.01$, Mann–Whitney) and Rapa (0.4 ± 0.01 mm^2, $P=0.03$, Mann–Whitney). Combination therapy failed to attenuate medial fibrosis (0.42 ± 0.19 mm^2, $P=0.08$, Mann–Whitney) significantly. Although a prominent neo-adventitia was apparent on microscopic examination, it was impossible for adventitial ECM content to be measured accurately, due to the uncertainty of the true adventitial boundary in a free peritoneal graft. Adventitial ECM (Sirius-red staining) density did, however, correlate inversely with vessel diameter ($r=-0.5$, $P=0.1$, Spearman's rank, data not shown).

Results of RT-PCR

There were significant reductions in the expression of TIMP-1 and collagen III, as well as significant increases in MMP-9 and TGF-β in untreated allografts relative to isografts (Fig. 3). MMP-9 and TGF-β transcript level were inhibited by both Rapa and CyA in isolation; however, this effect was not apparent in the combined

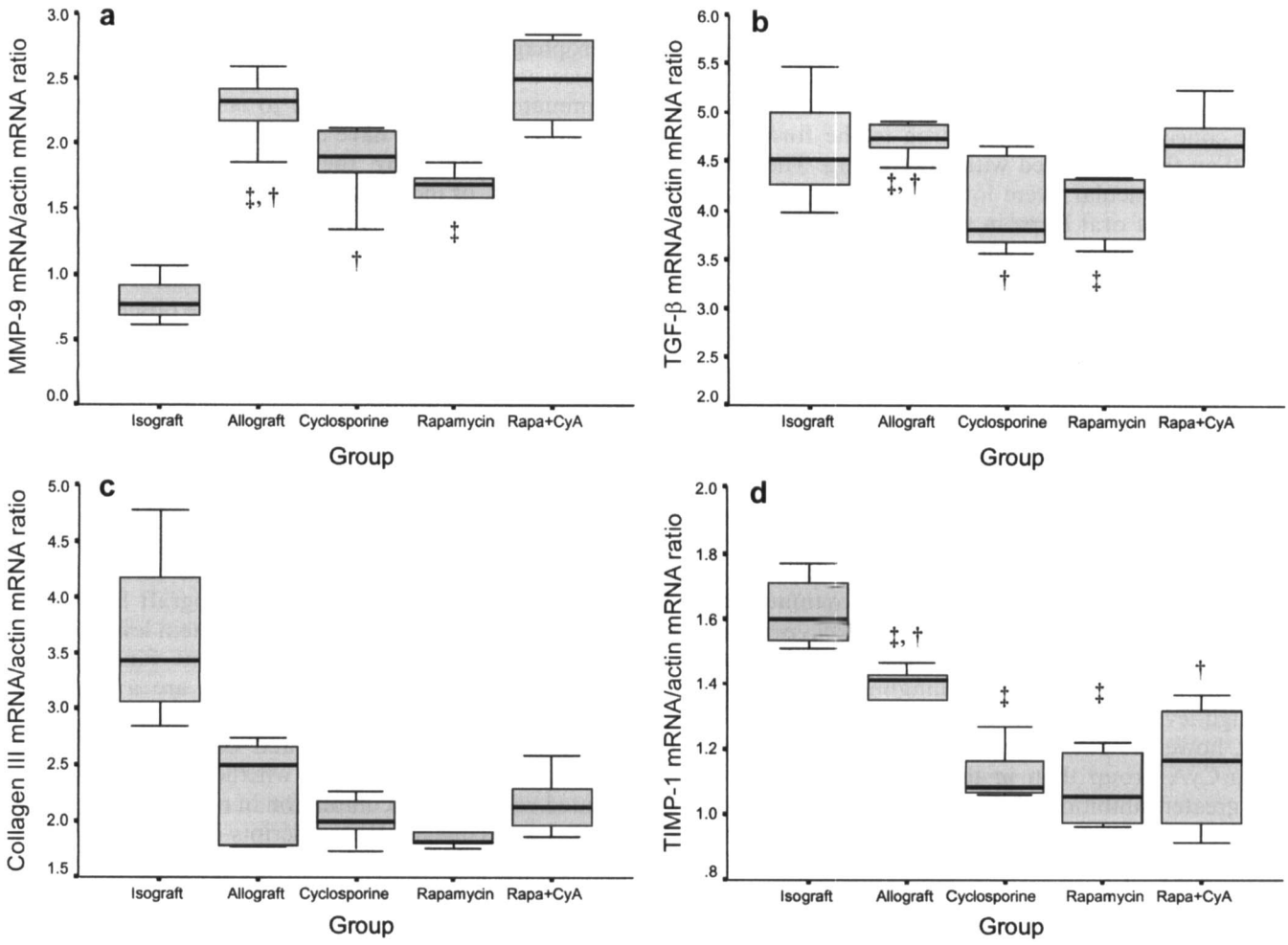

Fig. 3a–d Gene expression at 16 weeks. Ratios of ELISA values of RT-PCR cDNA product of mRNA species isolated from rat aorta using Dynabead extraction at 16 weeks. *Boxes* represent median plus SD, *whiskers* represent 95% CI, ‡ $P = 0.01$, Mann-Whitney test. **a** † $P = 0.04$, **b** † $P = 0.002$, **d** † $P = 0.008$, all Mann–Whitney

-treatment group. The observed changes in allograft collagen-III transcript levels were not significantly attenuated by immunosuppression. TIMP-1 mRNA levels were significantly attenuated by CyA and Rapa monotherapy, but not by combined treatment relative, to the positive control. There was, however, no difference between the CyA, Rapa or CyA/Rapa group mRNA transcript levels for collagen 3 or TIMP-1. There were no differences between any of the groups for TIMP-2 and TIMP-3, although TIMP-3 expression negatively correlated with TIMP-1 ($r = -0.6$, $P < 0.0001$) and collagen-III expression ($r = -0.8$, $P < 0.0001$, Spearman's rank, data not shown).

Discussion

These data demonstrate that Rapa and CyA in combination, at doses ten-times and three-times lower than either Rapa or CyA monotherapy, respectively, was as effective as either agent alone at preventing the intimal thickening in rat aortic allografts . Medial fibrosis, expansive remodelling, vessel inflammation and changes in the expression of fibrosis-associated genes (MMP-9 and TGF-β) were not inhibited by combination therapy at the doses administered, however.

In animal studies in vivo, CyA and Rapa demonstrate synergistic properties. Sub-therapeutic doses of Rapa (0.01–0.04 mg/kg per day) and CyA (0.5–2 mg/kg per day) prolonged rat cardiac and kidney allograft survival when compared with either drug alone or the additive effect of a combination of both [15]. There was no statistically significant difference in intimal development between Rapa or CyA monotherapy and that of the two in combination at doses ten-times and three-times lower than those of Rapa and CyA, respectively. This suggests that Rapa and CyA may act synergistically to inhibit intimal hyperplasia in this model. Synergy was not evident when other features of allograft vasculopathy were considered. The failure of combination therapy to attenuate MMP-9 or the inflammatory infiltrate suggests that these two agents at the dose reductions described

were not as efficacious at inhibiting alloimmune injury as was reported by Stepkowski et al. [15]. This discrepancy may be explained by the use of continuous i.v. infusions for drug administration, which produced higher steady-state concentrations of each drug in the former study [15] than those achieved with oral dosing. The doses of Rapa, in particular, were low, and given the poor bioavailability of oral Rapa in rats (15%), these results do not exclude a synergistic effect of reduced-dose CyA and Rapa on the fibroproliferative response in the presence of effective immunosuppression. In high-responder models the strength of the cellular immune response can obscure the relative contribution of other factors to the development of CAD, and the failure to suppress the inflammatory infiltrate is a weakness of this study. One possible interpretation of these results is that the measured histological and molecular changes may relate to the relative immunosuppressive efficacy of these drugs as opposed to a direct anti-fibroproliferative effect. That is, the attenuation of intimal hyperplasia, remodelling and ECM accumulation may represent a continuum of the immunosuppressant effect, with intimal hyperplasia in this model being suppressed by very low levels of immunosuppression and remodelling/medial fibrosis only by high levels. Such a pattern is not consistent with the data, however. There was less intimal thickening in the Rapa/CyA group than in the CyA-only group, which had greater inhibition of the inflammatory infiltrate and the greatest degree of intimal thickening among the treatment groups. This suggests that the effect of combination therapy on intimal hyperplasia is independent of the immunosuppressant effect. Similar inhibition of intimal hyperplasia in the absence of effective immunosuppression in rat cardiac allografts with low-dose Rapa monotherapy has been reported previously [16].

Both Rapa and CyA monotherapy significantly inhibited remodelling, medial ECM accumulation and the inflammatory infiltrate in rat aortic allografts. There was a more-severe level of inflammation in the Rapa/CyA group than in the Rapa and CyA monotherapy groups, and this was associated with higher MMP-9 and TGF-β transcript levels with combination therapy than with monotherapy. Previous studies on the F344-to-Lewis aortic allograft model have demonstrated that expansive vascular remodelling and medial ECM accumulation correlate with intra-graft MMP-9 transcript levels, which, in turn, are associated with severity of the inflammatory infiltrate [11]. Furthermore, suppression of the inflammatory infiltrate was associated with attenuation of MMP-9 expression, and it is our belief that the observed increases in gelatinase expression are derived from these cells. This is supported by observations that MMP-9 is expressed by infiltrating T cells and activated macrophages in vivo and in vitro [4] and also by studies in mice aortic allografts where MMP-9 is localised to inflammatory cells [8]. Failure to suppress this inflammatory infiltrate may have contributed to expansive remodelling directly, by the release of MMP-9 from macrophages, or indirectly, via the tissue response to the inflammatory injury. TGF-β (a potent pro-fibrotic growth factor) is also produced by inflammatory cells, and the higher levels in the positive-control and combined-treatment groups may underlie the observed ECM accumulation (fibrosis) in these two groups.

ECM accumulation results not only from the synthesis of ECM components but also by the inhibition of their breakdown by proteases. TIMP-1, a 28.5-kDa glycoprotein, is capable of inhibiting all activated collagenases, stromeolysins and gelatinases and has been implicated in the ECM accumulation that characterises a number of diseases [3]. TIMP-1 mRNA transcript levels in biopsies from renal transplants correlate with histomorphometric quantitation of allograft ECM content [13], whilst biopsy collagen-III protein levels, which accurately predict decline in transplant function and long-term kidney transplant survival, are a validated surrogate marker of renal allograft dysfunction [12]. We therefore measured TIMP-1 and collagen-III mRNA levels in an attempt to assess whether they may be associated with ECM accumulation in rat aortic allografts. TIMP-1 and collagen-III transcripts in positive controls were lower than in negative controls, which is at odds with the increased ECM accumulation seen in the vessel media of untreated allografts compared with isografts. Fibrosis in rat aortic allografts is, therefore, not associated with TIMP-1 expression (similarly to that in balloon-injured rat carotids [19]) and occurs due to the accumulation of ECM constituents other than collagen III (as is seen in balloon-injured pig and rat arteries [14, 18]. Separate processes may, therefore, regulate the ECM accumulation that occurs in blood vessels and that that occurs in the parenchyma of solid-organ allografts. This is also supported by the observation that the rate of interstitial fibrosis does not necessarily parallel the progression of allograft vasculopathy, as demonstrated in murine models of cardiac allograft dysfunction [1].

In conclusion, Rapa and CyA act synergistically to inhibit intimal hyperplasia in rat aortic allografts without attenuating the graft inflammatory infiltrate, medial fibrosis or expansive remodelling. Effective immunosuppression is required for the reduction of allograft fibrosis and vessel remodelling, which are associated with elevated intragraft MMP-9 and TGF-β but not TIMP-1 or collagen-III mRNA transcript levels.

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