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ORIGINAL ARTICLE

Combination of clopidogrel and everolimus dramatically reduced the development of transplant arteriosclerosis in murine aortic allografts

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Summary

Our group has shown that platelet inhibition with clopidogrel, an antagonist of the P2Y12 adenosine diphosphate receptor on platelets, reduced the formation of transplant arteriosclerosis. The aim of this study was to investigate whether a combination of cyclosporin or everolimus with clopidogrel has a beneficial effect on the development of transplant arteriosclerosis. Fully MHC mismatched C57Bl/6 ($H2^b$) donor aortas were transplanted into CBA.J ($H2^k$) recipients and mice received either clopidogrel alone (1 mg/kg/day) or in combination with cyclosporin (2 mg/kg/day) or everolimus (0.05 mg/kg/day). Grafts were analysed by histology and morphometry on day 30 after transplantation. In mice treated with clopidogrel alone, transplant arteriosclerosis was significantly reduced [intima proliferation 56 ± 11% vs. 81 ± 7% (control)/$n = 7$]. Daily application of everolimus reduced the development of transplant arteriosclerosis compared with untreated controls [intima proliferation of 29 ± 9% vs. 81 ± 7% (control)/$n = 7$]. Strikingly, combination of clopidogrel and everolimus almost abolished the formation of transplant arteriosclerosis [intima proliferation: 11 ± 8% vs. 81 ± 7% (control)/$n = 7$]. By contrast, combination of cyclosporin and clopidogrel compared with clopidogrel alone showed no additive effect. These results demonstrate that combination of platelet- and mammalian target of Rapamycin-inhibition can dramatically reduce the development of transplant arteriosclerosis.

Introduction

Progress in the development of immunosuppressive therapy has decreased the incidence of acute cellular and vascular rejection, but the development of transplant coronary arteriosclerosis continues to limit the survival of cardiac allograft recipients and is still the leading cause of late mortality after heart transplantation [1]. It frequently involves long segments of the affected arteries and is characterized by a diffuse and progressive thickening of the arterial intima in major coronary arteries and a stenotic medial disease affecting the small intramyocardial blood vessels [2,3]. This results in sudden or chronic progressive ischaemic damage to the transplanted heart with subsequent organ failure.

Numerous pathogenetic mechanisms have been suggested to be involved in the development of transplant arteriosclerosis, including immune mediated vascular injury, inflammation of the vascular endothelium, ischaemia reperfusion injury, cytomegalovirus infection and metabolic risk factors [2,4]. As interactions of platelets with the endothelium induce significant changes in the adhesive and chemotactic properties of endothelial cells that are able to trigger monocyte adhesion and transmigration resulting in an inflammatory reaction throughout the vessel wall [5,6], our recent interest has focused on

the involvement of platelets in the pathogenesis of transplant arteriosclerosis. Here we could recently show that monotherapy with clopidogrel effectively reduced the formation of transplant arteriosclerosis in a fully allogeneic murine aortic allograft model [7]. This effect was accompanied by reduced adhesion molecule expression within the recipients' serum and aortic transplant tissue associated with reduced infiltration of dendritic cells (DCs) and macrophages within the vascular wall. Therefore, we concluded that clopidogrel may have additional immunomodulating effects as treatment with anti-Glycoprotein-Ib (anti-GP-Ib) and anti-GP-VI monoclonal antibodies (mAbs) showed no significant difference in the amounts of transplant arteriosclerosis compared with untreated controls [8]. Moreover, two recent clinical studies have also highlighted that platelet activation is associated with the development and progression of transplant arteriosclerosis [9,10].

Clopidogrel, a member of the thienopyridines, has become an important therapeutic agent for patients with coronary heart disease [11] and has been shown to decrease the incidence of coronary artery stent thrombosis and to reduce myocardial infarction, stroke and vascular death within these patients [12]. It inhibits platelet activation by blocking an adenosine diphosphate (ADP) receptor on platelets, recently designated P2Y12 [13,14]. Clopidogrel is inactive *in vitro* and requires hepatic metabolism for production of its active metabolite [15].

As we have recently shown that monotherapy with clopidogrel effectively reduced the formation of transplant arteriosclerosis in a murine aortic allograft model [7] and that this effect was in part was caused by a reduction of macrophage and DC infiltration within the transplant [8], the aim of this study was to investigate if a combination of clopidogrel and a T-cell modulating agent such as a calcineurin antagonist (cyclosporin) and mammalian target of Rapamycin (mTOR) inhibitor (everolimus) or cyclosporin have beneficial effects on the development of transplant arteriosclerosis in a murine experimental aortic allograft model.

Materials and methods

Animals

Mice, C57Bl/6 ($H2^b$) and CBA.J ($H2^k$) were obtained from Charles River (Sulzbach, Germany) and aged between 6 and 12 weeks at the time of experimental use. They were bred and maintained at the central animal facility of the University of Erlangen-Nürnberg (Franz-Penzoldt-Zentrum) under specific pathogen-free conditions and treated in accordance with institutional and state guidelines.

Treatment protocols

Clopidogrel (Plavix®; Sanofi-Synthelabo, Berlin), was obtained from the local hospital pharmacy and 75 mg tablets were dissolved in 0.9% saline under sterile conditions. This solution was then diluted appropriately to receive a final daily clopidogrel dose of 1 mg/kg/day. As dissolved, clopidogrel is unstable [15] the clopidogrel solution was freshly prepared every day and injected intraperitoneally (i.p.) immediately after preparation (Group 1). Cyclosporin (Sandimmun; Novartis, Nuremberg, Germany) was also obtained from the local hospital pharmacy and animals were treated with a daily dose of 2 mg/kg/day (Group 3). Everolimus (Certican®; Novartis) was also purchased at the local hospital pharmacy and mice received a daily dose of 0.05 mg/kg/day. A combination of clopidogrel (1 mg/kg/day) and cyclosporin (2 mg/kg/day) (Group 4) respectively clopidogrel (1 mg/kg/day) and everolimus (0.05 mg/kg/day) (Group 5) was also investigated. Daily treatment of all drugs was started immediately after transplantation for 30 days as an i.p. injection.

Platelet aggregation

For *ex vivo* platelet aggregation analyses, blood samples (0.5 ml) were collected in 3.2% citrate and immediately processed after harvest. Platelet aggregation was evaluated by optical aggregometry at 37 °C using a 2-channel Chronolog aggregometer (Elvi Logos, Milan, Italy) as described earlier [7]. Maximal aggregation was mostly seen around 5 min and was used as measurement of aggregation.

Plasma concentration of cyclosporin and everolimus

Cyclosporin (Sandimmun®) plasma concentrations were measured in the Department of Hematology and Oncology's routine laboratory using a fluoroscopic polarization immunoassay (TDx/TDxFLx® Cyclosporin Monoclonal Whole Blood Assay; Abbott Laboratories, Abbott Park, IL, USA). Everolimus (Certican®) plasma concentrations were also measured using a fluoroscopic polarization immunoassay (INNOFLUOR®-CERTICAN®-Assay; Seradyn, Indianapolis, IN, USA). For both analyses, each 150 µl of mouse blood was harvested from the plexus retroorbitalis mixed with 50 µl ethylene diamine acetic acid (EDTA) and immediately transferred for analysis. Every sample was analysed in triplicates.

Abdominal aortic transplantation

The procedure was performed using a modified technique initially described by Koulack *et al.* [16]. In brief, the donor thoracic aorta was isolated, resected and

transferred to the recipient animal. The recipient aorta was clamped and then transected with sharp microvascular scissors. A proximal end to end anastomosis was performed. The aortic graft was then repositioned and the anastomosis continued with single interrupted sutures.

Analysis of the aortic graft

Aortic grafts were removed under anaesthesia on day 30 after transplantation. Grafts were perfused with saline and were flash frozen in optimal cutting temperature (OCT) medium (Tissue-Tek®; Sakura, The Netherlands) in liquid nitrogen for morphometric analysis of 7 μm cryostat sections. A minimum of five transverse sections was analysed from each graft.

Morphometry

Five sections from each graft harvested at day 30 were stained with Elastin/van Gieson and analysed at an original magnification of ×200 using a conventional light microscope. A digitized image of each section was captured and the areas within the lumen and the internal elastic lamina were circumscribed manually and measured as previously described [17]. All image analyses were carried out on a colour display monitor using ANALYSIS® Image Analysis software (Olympus, Hamburg, Germany).

Statistical analysis

Results are given as the mean per group ± SD which was derived from the mean per graft. Statistical analysis was performed using a one-way ANOVA followed by a Bonferroni correction. Differences between groups are considered as significant when $P < 0.05$.

Results

Monitoring of plasma levels of everolimus, cyclosporin and platelet aggregation

We have previously shown that treatment with a standard 'human dose' of 1 mg/kg clopidogrel resulted in impaired platelet function and attenuated the formation of transplant arteriosclerosis in an experimental mouse aortic transplant model, therefore this dose was used for the study [7]. To determine the accurate everolimus and cyclosporin dosing regimen, mice ($n = 7$ per group) were treated daily with different doses of cyclosporin (10 mg/day, 2 mg/day, 1 mg/day) or everolimus (0.1 mg/day, 0.05 mg/day, 0.03 mg/day) or a combination of both drugs with clopidogrel. Plasma levels for everolimus were aimed at 3–9 ng/ml and for cyclosporin at 150–300 ng/ml. Blood analysis was prepared on days 4, 8 and 14. For cyclosporin the most suitable dose was 2 mg/kg/day and for everolimus 0.05 mg/kg/day resulting in appropriate blood target levels of the respective drug throughout the experimental protocol. Higher doses were not tolerated well by the mice and lead to death or indisposition of the mice. In addition, blood from clopidogrel treated mice showed impaired platelet function and was previously published by our group [7]. Table 1 summarizes blood levels of everolimus and cyclosporin (ng/ml) and platelet aggregation (%) after treatment with clopidogrel at different timepoints throughout the experimental protocol.

Table 1. Blood levels of everolimus and cyclosporin and platelet aggregation after treatment with clopidogrel at different timepoints.

	Day 8	Day 14	Day 12
Everolimus (ng/ml)	13 ± 2	12 ± 2	9 ± 1
Cyclosporin (ng/ml)	434 ± 44	489 ± 44	425 ± 74
Clopidogrel (%)	31 ± 16	23 ± 12	19 ± 4
Clopidogrel (%) +	3 ± 11	27 ± 9	21 ± 6
Everolimus (ng/ml)	11 ± 2	10 ± 2	7 ± 2
Clopidogrel (%) +	28 ± 12	17 ± 7	18 ± 9
Cyclosporin (ng/ml)	392 ± 49	420 ± 93	332 ± 54

Monotherapy with clopidogrel or everolimus resulted in reduced levels of transplant arteriosclerosis

Aortic allografts (C57Bl/6 (H-2^b) from recipients (CBA/J (H-2^k) were analysed 30 days after transplantation, the time point at which distinctive changes of transplant arteriosclerosis are most evident [18]. When recipient mice were treated with clopidogrel (1 mg/kg/day) in the absence of any other immunosuppression transplant arteriosclerosis was significantly reduced when compared with untreated controls [intimal proliferation 56 ± 11% (1 mg/kg clopidogrel) vs. 81 ± 7% (control) $n = 7$ per group $P \leq 0.05$] (Figs 1b and 2). Recipients treated daily with 0.05 mg/kg everolimus showed significantly reduced transplant arteriosclerosis [intimal proliferation 29 ± 9% (0.05 mg/kg everolimus) vs. 81 ± 7% (control) $n = 7$ per group $P \leq 0.05$] (Figs 1c and 2). However, after monotherapy with cyclosporin (2 mg/kg/day), there was no effect on vascular lesion formation detectable when compared with control [intimal proliferation 72 ± 9% (2 mg/kg cyclosporin) vs. 81 ± 7% (control) $n = 7$ per group $P \leq 0.05$] (Figs 1d and 2). Syngeneic controls [C57Bl/6 ($H2^b$) into C57Bl/6 ($H2^b$) recipients] did not show any signs of transplant arteriosclerosis at 30 days after transplantation, indicating that non-immunological mecha-

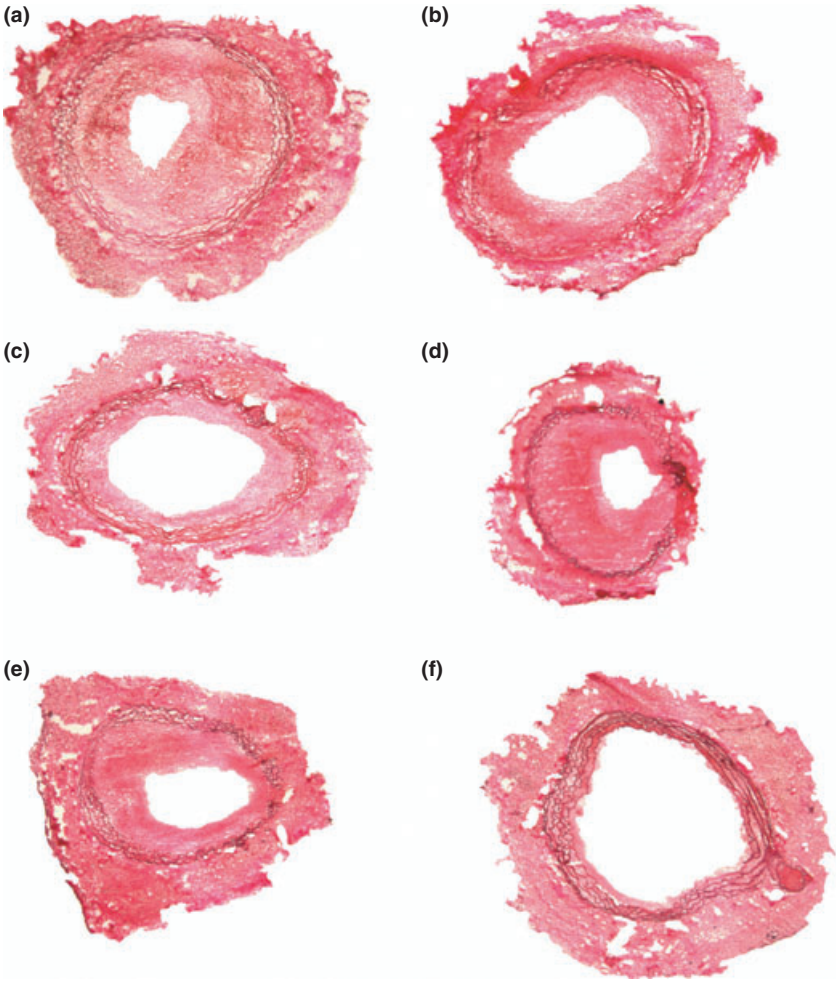

Figure 1 Histopathological evaluation of the morphology of untreated fully allogeneic C57Bl/6 aortic grafts implanted into CBA recipients showed high levels of intimal proliferation (a) whereas C57Bl/6 aortic grafts of recipients treated with 1 mg/kg clopidogrel (b), 0.05 mg/kg everolimus (c) showed reduced intimal proliferation 30 days after transplantation. Recipients treated with 2 mg/kg cyclosporin (d) and the combination of 1 mg/kg clopidogrel + 2 mg/kg cyclosporin (e) showed no effect. However, grafts of recipients treated with the combination of 1 mg/kg clopidogrel + 0.05 mg/kg everolimus showed almost no intimal proliferation (f). The tissue was snap-frozen and representative sections were stained with Miller's Elastin/van Gieson stain. Pictures were taken at an original magnification of ×200.

nisms alone were not sufficient to initiate the formation of transplant arteriosclerosis in this model (data not shown).

Combined treatment with clopidogrel and everolimus dramatically reduced the development of transplant arteriosclerosis

Surprisingly, daily administration of clopidogrel (1 mg/kg) and everolimus (0.05 mg/kg) dramatically reduced the formation of any vascular lesions leading to transplant arteriosclerosis [11 ± 8% (clopidogrel and everolimus) vs. 29 ± 9% (everolimus alone) vs. 56 ± 11% (clopidogrel alone) vs.81 ± 7% (untreated control) $n = 7$ per group $P \leq 0.05$] (Fig. 1f and 2). By contrast, combination of clopidogrel (1 mg/kg) and cyclosporin (2 mg/kg) had no beneficial effect on the formation of transplant arteriosclerosis [71 ± 12% (clopidogrel and cyclosporin) vs. 56 ± 11% (clopidogrel alone) vs. 81 ± 7% (untreated control)] (Figs 1d,e and 2).

Reduced intragraft expression of ICAM-1 and CD40L mRNA after combined treatment with clopidogrel and everolimus

As we have previously shown that recipient-treatment with clopidogrel affected levels of circulating sCD40L and inter-cellular adhesion molecule-1 (ICAM-1) and E-selectin production within the graft tissue [8], we investigated whether a combination of clopidogrel (1 mg/kg) and everolimus (0.05 mg/kg) has an impact on the expression of these cytokines within the graft. Intragraft cytokine mRNA was measured 14 days after transplantation, a time point when changes in cytokine expression are most prominent, as shown earlier [18]. Aortic allografts from recipients treated with clopidogrel (1 mg/kg) and everolimus (0.05 mg/kg) exhibited significantly decreased intragraft mRNA expression of ICAM-1 (9.1-fold) and CD40L (3.0-fold) compared with untreated controls and monotherapy with everolimus (Fig. 3a and c). However, E-selectin mRNA expression was significantly reduced compared with untreated controls (7.0-fold) after

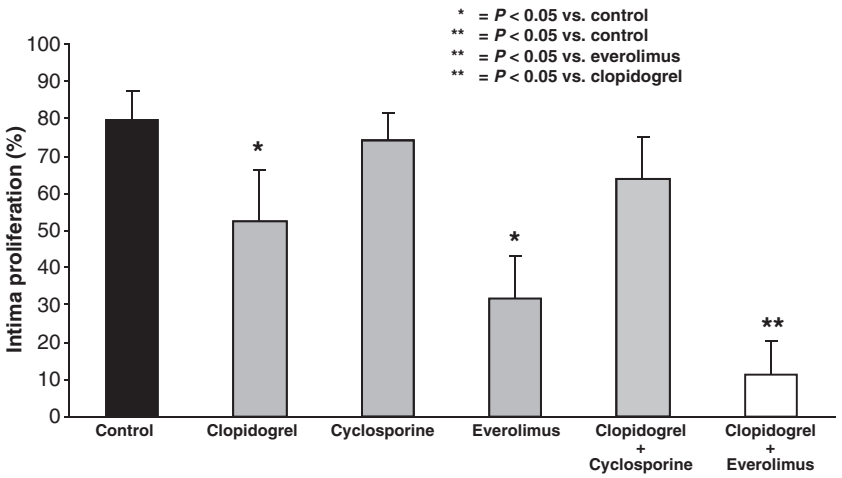

Figure 2 For the morphometric analysis of the degree of intimal thickening Miller's Elastin/van Gieson stained sections were used. Areas within the lumen and the internal elastic lamina were circumscribed manually and measured. From these measurements a quotient for the thickness of the intima (Q_{int}) was calculated. Q_{int} indicates the relative thickness (%) of the intima. Five measurements from different areas of each aortic graft were obtained for this analysis. (n = 7 animals per group/ P-values as indicated in the diagram).

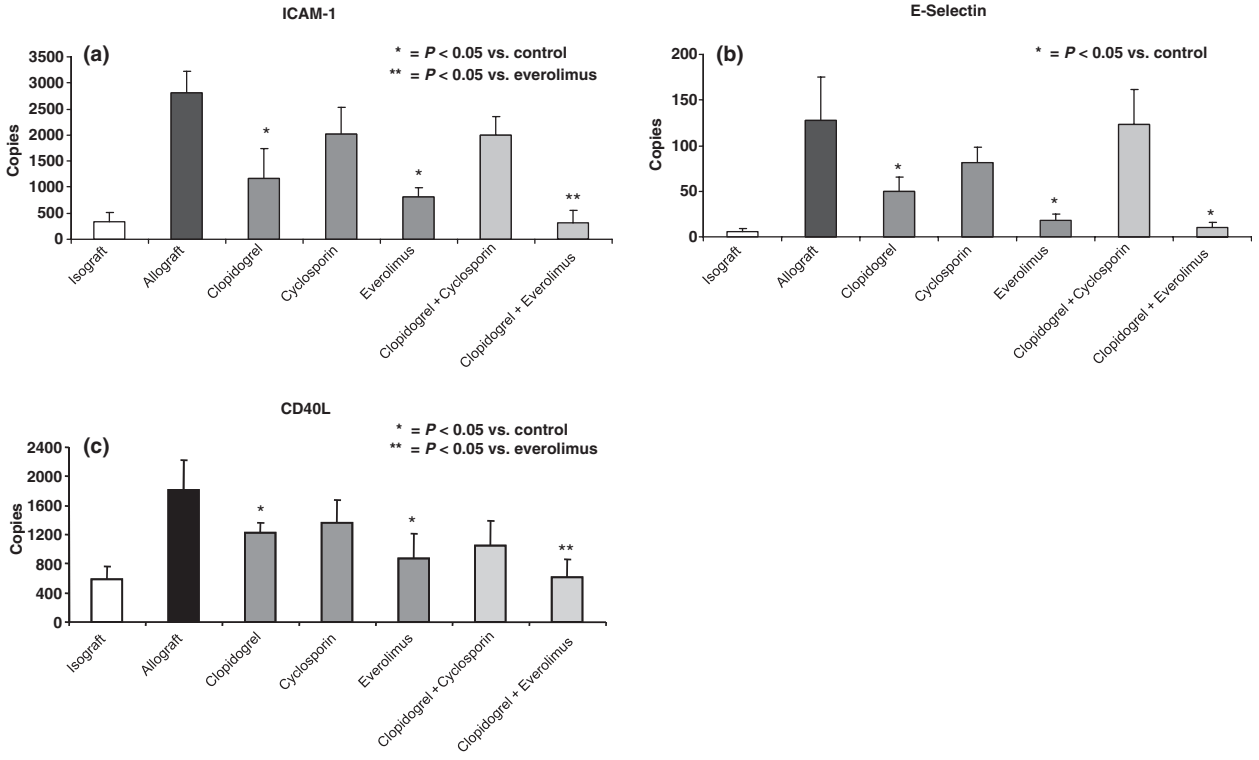

Figure 3 Quantitative RT-PCR analysis of intragraft ICAM-1, E-selectin and CD40L mRNA expression. C57Bl/6 ($H2^b$) aortic grafts implanted into CBA/J ($H2^k$) recipients were analysed 14 days after transplantation, the timepoint of maximum cellular infiltration. Analysis was performed for ICAM-1 (a), E-selectin (b) and CD40L (c) mRNA. (n = 7 animals per group/P-values as indicated in the diagram).

combined treatment with everolimus and clopidogrel, but did not show a more profound effect when compared with monotherapy with everolimus (Fig. 3b). Analysis of baseline cytokine gene expression in isografts was characterized by low levels of ICAM-1 and E-selectin and moderate levels of CD40L mRNA (Fig. 3).

Discussion

The development of transplant arteriosclerosis in cardiac allografts after transplantation is a multifactorial process with macrophages, T cells, pro-inflammatory cytokines, adhesion molecules, growth factors and alloantibodies

implicated in both initiation and progression of this chronic inflammatory process [19,20]. We have previously shown that platelets do play an important role in chronic rejection with its hallmark feature transplant arteriosclerosis as monotherapy with clopidogrel effectively reduced the formation of transplant arteriosclerosis in a murine aortic allograft model [7]. This effect was in part caused by a reduction of macrophage and DC infiltration within the transplant [8]. Here we can show for the first time that combination of clopidogrel and the mTOR inhibitor everolimus dramatically reduced the development of transplant arteriosclerosis in a mouse aortic allograft model. By contrast, application of clopidogrel and the calcineurin inhibitor cyclosporin revealed no additional beneficial effect.

To determine the most suitable and effective daily dose for cyclosporin and everolimus, a range of dose finding experiments were undertaken and administration of 2 mg/kg/day cyclosporin and for 0.05 mg/kg/day everolimus resulted in appropriate plasma levels within the recipient mice, comparable with human blood target levels of the respective drug and similar to earlier studies [21–23].

Everolimus is meanwhile an established drug in organ transplantation [24–26] and inhibits mTOR in proliferating T cells. Thereby, everolimus prevents proliferation and clonal expansion of antigen-activated T-cells, a process triggered by T-cell-specific interleukins like IL-2 and IL-15. Experimental studies have shown that everolimus inhibits smooth muscle cell proliferation, prevents neointimal thickening and delays the development of transplant arteriosclerosis in rat cardiac [21,23,27–29] and carotid allografts [21]. In addition, everolimus has been shown to delay the onset of chronic rejection in rat renal allografts [30]. Earlier on, sirolimus was able to prolong survival and delay the progress of transplant arteriosclerosis in experimental rat cardiac allograft transplantation [31–33]. Data of the current study show that monotherapy with everolimus significantly reduced neointima formation within the aortic allograft, which is in accordance with the previous experimental data mainly recovered in rat models [21,23,27,29]. In clinical heart transplantation, everolimus reduced acute rejection and delayed the formation of transplant arteriosclerosis; however, long-term results are still pending as the follow up is 4 years by now [34].

Leucocytes and platelets can both induce enhanced surface expression of ICAM-1 [35] and E-selectin [36] on endothelial cells that mediate adhesion and transmigration of monocytes and leucocytes, an essential step during the development of transplant arteriosclerosis [37]. In accordance with this concept, treatment with everolimus resulted in a significant reduction of ICAM-1 and E-selectin. In addition, CD40L expression within the aortic graft was also reduced as everolimus has been shown to prevent proliferation and clonal expansion of antigen-activated T-cells.

Cyclosporin binds to the cytosolic protein cyclophilin [38] of immunocompetent lymphocytes, especially T-lymphocytes. This complex of cyclosporin and cyclophilin inhibits calcineurin [39], which is responsible for activating the transcription of interleukin 2 [40]. It also inhibits lymphokine production and interleukin release and therefore leads to a reduced function of effector T-cells. Numerous clinical and experimental studies exist about the effects and function of cyclosporin. In rat cardiac allografts cyclosporin was able to up-regulate the expression of tissue growth factor beta (TGF-β) thereby leading to increased transplant arteriosclerosis [41–44]. Earlier on Koskinen *et al.* could show that therapy with Cyclosporin leads to endothelialitis and accelerated arteriosclerosis in rat aortic allografts [45]. Data of the current study also reveal that monotherapy with cyclosporin is not effective in preventing the formation of transplant arteriosclerosis. One explanation may be a calcineurin-independent and cyclosporin insensitive pathway of IL-2-production via activation of c-Jun N-terminal kinase (JNK) [46] and nuclear factor kappa B (NF-kB) [47] by protein-kinase C.

The most striking and surprising result of the study was the fact that combined treatment with everolimus and clopidogrel dramatically reduced the development of transplant arteriosclerosis, implying a synergistic effect of both drugs. Everolimus inhibits the proliferation and clonal expansion of antigen-activated T-cells impairing the interaction with DCs and macrophages, which on the other hand are modulated by clopidogrel [8]. Therefore, signalling that leads to activation of T cells, macrophages and DCs is blocked from both sides. This view is supported by the finding that a combination of both drugs resulted in even more decreased intragraft expression of ICAM-1 and CD40L compared with monotherapy with either everolimus or clopidogrel in the current study. In addition, data from a carotid artery transplant model suggest that CD40L and P-selectin facilitate ICAM-1 expression on the surface of endothelial cells making it easier for monocytes to transmigrate [48]. Finally, as already mentioned, enhanced surface expression of ICAM-1 [35] and E-selectin [36] on endothelial cells mediates adhesion and transmigration of monocytes and leucocytes, an essential step during the development of transplant arteriosclerosis [37]. Encouraged by these experimental results, a multicentre study investigating the clinical potential of platelet inhibition in addition to mTOR inhibition after heart transplantation is about to start throughout Germany.

By contrast, combination of clopidogrel and cyclosporin did not show a beneficial effect at all on the development of transplant arteriosclerosis and in fact abolished the beneficial effect of clopidogrel. As this result was somewhat surprising, our first suspicion was that both drugs compete for the P450 cytochrome and therefore clopidogrel was not converted in its active metabolite sufficiently resulting in unimpaired platelet function. However, platelet aggregation in this experimental group revealed impaired platelet function, suggesting that clopidogrel was metabolized and effective. At this stage, we do not have a further explanation for this result, but experiments investigating this finding are currently ongoing in our laboratory.

In conclusion, we have shown that the combination of clopidogrel and everolimus can dramatically reduce the development of transplant arteriosclerosis in an experimental murine aortic allograft model. Both clopidogrel and everolimus are readily available drugs with an established clinical safety profile. Therefore, our findings have important clinical implications as patients suffering from transplant arteriosclerosis after cardiac transplantation may benefit from this new treatment strategy.

Authorship

S.A.-O., M.W. and S.M.E.: designed the study. S.E., C.H., J.H. and M.R.-G.: performed the experiments and collected data. S.E., S.A.-O. and S.M.E.: analysed the data. S.E., S.A.-O., M.W. and S.M.E.: wrote the paper.

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