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ORIGINAL ARTICLE

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Two good reasons for an angiotensin-II type 1 receptor blockade with losartan after cardiac transplantation: reduction of incidence and severity of transplant vasculopathy

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Abstract Despite considerable progress in immunosuppressive therapy, the incidence and severity of transplant vasculopathy (TVP) after cardiac transplantation have not declined. The renin–angiotensin system (RAS) plays a pivotal role in the proliferation of vascular smooth muscle cells (VSMCs) contributing to TVP. We compared the effects of an angiotensin-II blocker, losartan (AT_1 blocker), and an angiotensin-converting enzyme (ACE) inhibitor, enalapril, on the incidence of diseased vessels and the severity of experimental TVP in the Lewis-to-Fischer rat heterotopic heart transplantation model. Recipients were randomly divided into six groups, group 1: no therapy, group 2: 3 mg/kg per day cyclosporine (CyA) s.c., group 3: CyA and 10 mg/kg per day losartan p.o., group 4: CyA and 40 mg/kg per day enalapril p.o., and groups 5 and 6: as groups 3 and 4, but additionally pre-treated with losartan or enalapril 7 days prior to transplantation. Eighty days after grafting, we assessed the incidence and severity of TVP, expressed as percentage of diseased vessels and mean vessel occlusion (MVO), by digitizing morphometry. CyA and CyA/enalapril post-treatment significantly reduced MVO, compared with controls, but not the incidence. Additional reduction of MVO was achieved in CyA/enalapril pre-treatment and both CyA/losartan pre- and post-treatment groups when compared with CyA and untreated controls. However, only losartan post-treatment in combination with CyA reduced both incidence and MVO. Our results validate the important role of the RAS in neointimal proliferation after cardiac transplantation. Losartan appears to be superior to enalapril in preventing TVP after experimental cardiac transplantation. Therefore, AT_1 blockade with losartan might be a therapeutic option for the prevention of TVP in human heart recipients.

Keywords Angiotensin-converting enzyme · Renin–angiotensin system · AT_1 receptor blockade · Transplant vasculopathy

Introduction

The renin–angiotensin system (RAS) plays a central role in the homeostasis of blood pressure and plasma volume, but in the past 10 years evidence has indicated that angiotensin II (Ang II), a central component of the RAS, also participates in the development of vascular smooth muscle cell (VSMC) proliferation, a pathological status that often occurs as a response to injury. Ang II can promote muscle cell growth and cardiovascular hyper-

trophy and can contribute to the development of neointimal proliferation in vascular structures after coronary angioplasty or organ transplantation [28, 34].

Ang II, after it is converted from angiotensin I by the angiotensin-converting enzyme (ACE), can interact at least with two main classes of Ang-II receptors: the Ang-II type 1 receptor (AT_1) and the Ang-II type 2 receptor (AT_2) [10]. Both receptors are located on endothelial cells and VSMCs [36, 14]. The coupling of Ang II to the AT_1 receptor induces growth effects in several tissues through the involvement of at least five effector systems: phospholipase C [5], D [24], A2 [2], adenylate cyclase [16], and calcium channels [35]. Ang II may also exert anti-proliferative effects via the stimulation of AT_2 receptors [9, 39]. Although the dominant receptor type on VSMCs is the AT_1 receptor [2, 24], AT_2 receptors are re-expressed in neointimal formation after vascular injury [15, 32, 40]. Both receptor types and the production of ACE, responsible for Ang-II formation, are up-regulated after vascular injury and may, therefore, contribute to the pathological status of restenosis and transplant vasculopathy (TVP) after transplantation [26]. Ang II induces the expression of adhesion molecules in human endothelial cells and activates human monocytes, resulting in increased adhesion to human endothelial cells [13]. Ang II by itself is chemotactic for T lymphocytes [8, 41]. In animal studies, ACE inhibitors [31] and AT_1 receptor blockers [20] have been shown to reduce neointimal formation in many experimental models of restenosis. Kobayashi et al. reported a reduction of TVP in a rat cardiac transplant model using captopril and pointed out that the RAS may also play a role in transplant arteriosclerosis [23]. Therefore, the purpose of our study was to assess the effects of losartan, an Ang-II (AT_1) receptor antagonist, in comparison with enalapril, an ACE inhibitor, on the incidence of diseased vessels and severity of TVP using a heterotopic cardiac model of transplantation from Lewis-to-Fischer rats.

Materials and methods

Experimental model

All animals received humane care in compliance with the *Principles of laboratory animal care* formulated by the Institute of Animal Resources and the *Guide for the care and use of laboratory animals* prepared by the Institute of Laboratory Animals Resources and published by the National Institutes of Health (NIH Publication No.86–23, revised 1985) and animal experiment regulations at our local institute.

The Lewis-to-Fischer rat heterotopic heart transplant model is a major-histocompatibility-matched, minor-histocompatibility-mismatch combination characterized by the development of TVP, which appears morphologically similar to the human disease [1]. Inbred male F344 rats and male Lewis rats weighing 180–220 g were obtained from Harlan and housed in stainless-steel cages with controlled light/dark cycles and ad libitum access to food and water.

Heterotopic heart transplantation

Donors and recipients were anesthetized with sodium pentobarbital before surgery. Heterotopic transplantation was carried out from Lewis (donor) to Fischer (recipient) rats and was performed via a modified technique according to Ono and Lindsey [29]. The aorta and pulmonary artery of the donor heart were anastomosed to the recipient's infrarenal abdominal aorta and inferior cava by a running-suture technique [33]. Graft viability was assessed by daily palpation and the function evaluated on a scale of 0–4, with 4 representing a normal heartbeat and 0 the absence of mechanical activity. Rejection was clinically defined as cessation of a detectable heartbeat.

Drug preparation and treatment

Losartan and enalapril – a generous gift from MSD (Munich, Germany) – were dissolved in water and administered orally at a concentration of 10 mg/kg per day or 40 mg/kg per day, respectively. To enhance the effects of enalapril and losartan treatment on the development of TVP after allografting, we treated two groups of recipients 7 days prior to transplantation, and treatment was continued until the animals were sacrificed. In all groups, enalapril or losartan therapy was continued for 80 days after transplantation, when the rats were killed. To reduce graft loss during study time, all animals were treated with low-dose cyclosporine (3 mg/kg per day s.c.). One group received only cyclosporine (CyA), and the control group received no therapy, neither CyA nor enalapril nor losartan.

Study groups

The study groups were as follows:

1. Untreated controls ($n = 12$).
2. Animals receiving only 3 mg/kg per day CyA ($n = 12$).
3. Animals post-treated with losartan ($n = 12$): 10 mg/kg per day losartan + 3 mg/kg per day CyA.
4. Animals post-treated with enalapril ($n = 12$): 40 mg/kg per day enalapril + 3 mg/kg per day CyA.
5. Animals pre-treated with losartan ($n = 12$): recipients and donors received 10 mg/kg per day losartan 7 days prior to transplantation. After transplantation, animals received 10 mg/kg per day losartan + 3 mg/kg per day CyA.
6. Animals pre-treated with enalapril ($n = 12$): recipients and donors received 40 mg/kg per day enalapril 7 days prior to transplantation. After transplantation, animals received 40 mg/kg per day enalapril + 3 mg/kg per day CyA.

Histological examination

Animals were randomized to six study groups of 12 animals each and were killed 80 days after transplantation. The transplanted hearts were fixed with glutar-paraformaldehyde, embedded in paraffin, and stained with hematoxylin–eosin for scoring of acute rejection or domac elastica for analysis of neointimal proliferation. Four sections of each heart were examined by standard light microscopy, and all arteries in a given section were viewed separately with high magnification. Grading of cellular rejection was performed according to the system of the International Society of Heart and Lung Transplantation (ISHLT) [6] as follows: 0, no evidence of acute rejection; Ia, focal (perivascular or interstitial) infiltrate; Ib, diffuse but sparse infiltrate; II, one focus only, with

aggressive infiltration and/or focal myocyte damage (focal moderate rejection); IIIa, multifocal aggressive infiltrates (low moderate rejection); IIIb, diffuse inflammatory process (borderline/severe); IV, diffuse, aggressive polymorphous ± edema, ± hemorrhage, ± vasculitis.

The extent of neointimal proliferation in coronary arteries was assessed by digitizing morphometry, with a KS300 System with a Zeiss Axioplan microscope (Zeiss, Germany) and expressed as the MVO of all viewed arteries, in percent. Vessels in the middle of fibrotic areas or immediately adjacent to fibrous scar tissue were excluded from analysis.

Definition of cardiac allograft vessels

Vessels with a well-defined smooth muscle cell layer in the vasculature wall and a lamina elastica interna were identified as arteries. Epicardial arteries were defined as arteries with a luminal diameter of more than 50 μm and with a thick vessel wall. Intramyocardial arteries were defined as arteries with a luminal diameter of less than 50 μm and a thin vessel wall.

Incidence of diseased vessels with TVP

The incidence of diseased vessels with TVP in a given section was calculated as:

$$\frac{\text{Number of vessels with signs of neointimal proliferation}}{\text{Total number of vessels in section}} \times 100 = \text{incidence\%} \quad \text{(a)}$$

Morphometry of TVP – mean vessel occlusion

For measurement of TVP (myointimal proliferation) in arteries, the area of the neointima and the area of the remaining lumen of the vessel were deteremined. The occlusion was calculated as follows:

$$\frac{\text{A – neointima}}{\text{A – neointima} + \text{A – remaining lumen}} \times 100 = \text{vessel occlusion\%} \quad \text{(b)}$$

(A: area)

All arteries in a paraffin section were measured, and vessel occlusion was calculated as MVO.

Cyclosporin A levels

Cyclosporin A levels were determined 50 days after transplantation and at death, with the EMIT Kit. The data are expressed as mean levels in nanograms/milliliter. Twelve hours after application of CyA, blood samples were collected by puncture of the retro-orbital venous plexus into EDTA vials (0.5 ml).

Statistical analysis

All data are expressed as mean ± standard deviation and, in cases of acute rejection score, as median. One-factor analysis of variance (ANOVA) was performed to assess the statistical significance of differences among the study groups, in terms of morphometric and other parameters (incidence and CyA levels). Whenever a statistically significant difference was detected by ANOVA, the Scheffe multiple-comparison procedure was performed to determine which inter-group differences were statistically significant. P values of less than 0.05 were considered significant.

Results

Graft survival

The mean ischemia time was 22 ± 3 min with an overall success rate greater than 95%, and there was no significant difference between the groups. During the study, all rats gained weight, with no statistically significant differences in the percentage of weight gain between the groups (mean 38 ± 5%). All cardiac graft recipients survived for 80 days after transplantation.

Acute rejection

Eighty days after cardiac grafting, the median of the acute rejection scores was determined with ISHLT IV in untreated animals and with ISHLT IIIa in all other groups. There was no statistically significant difference in acute rejection scores between enalapril and losartan pre- and post-treated animals and between the CyA- and ACE-/AT_1-blocker-treated animals ($P > 0.9$). When compared with the untreated control animals, the reduction of acute rejection was statistically significant ($P < 0.05$).

Cyclosporin A levels

Within the cyclosporin A-treated groups there was no statistically significant difference in resulting CyA levels (Fig. 1). The levels ranged from 302.25 ± 47 to 395.72 ± 98 ng/ml.

Incidence of diseased vessels with TVP

In untreated control animals, an incidence of 97.9 ± 4.1% was observed 80 days after transplantation (Fig. 2). With CyA therapy, the incidence was reduced to an extent of 78.1 ± 12.3%, but this reduction was not statistically significant when compared with untreated control animals. Treatment with CyA and enalapril starting immediately after transplantation (post-treated) lowered the incidence to 69.3 ± 14%, which was significant when compared with untreated controls, but not significant when compared with CyA-treated animals.

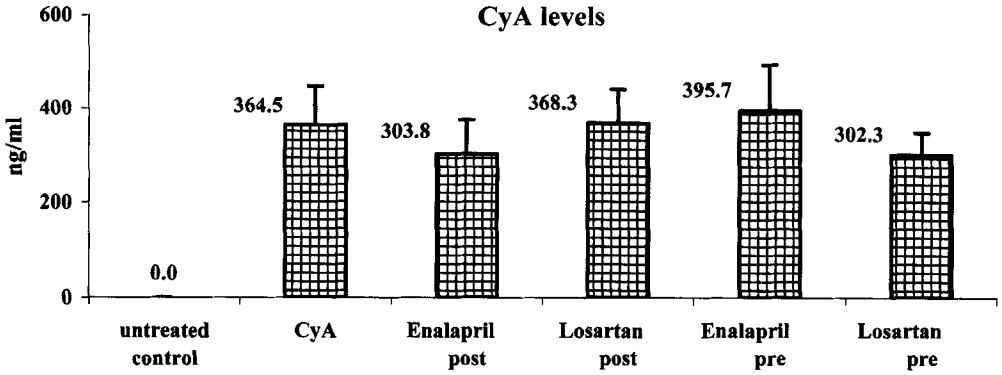

Fig. 1 CyA levels

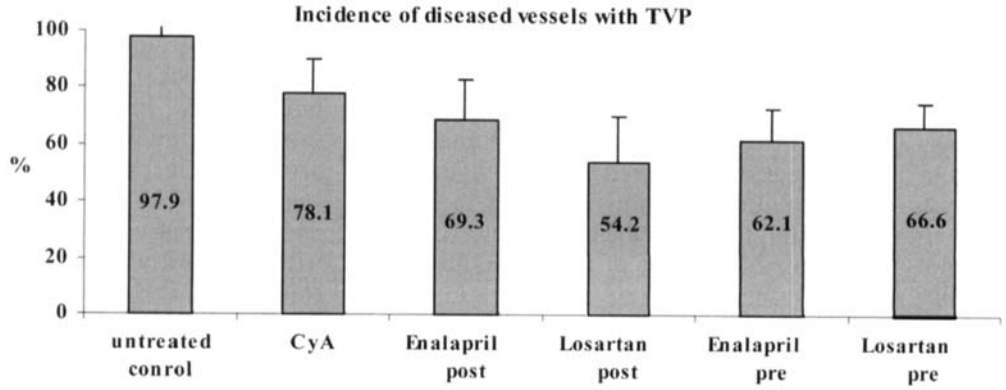

Fig. 2 Incidence of diseased vessels with TVP

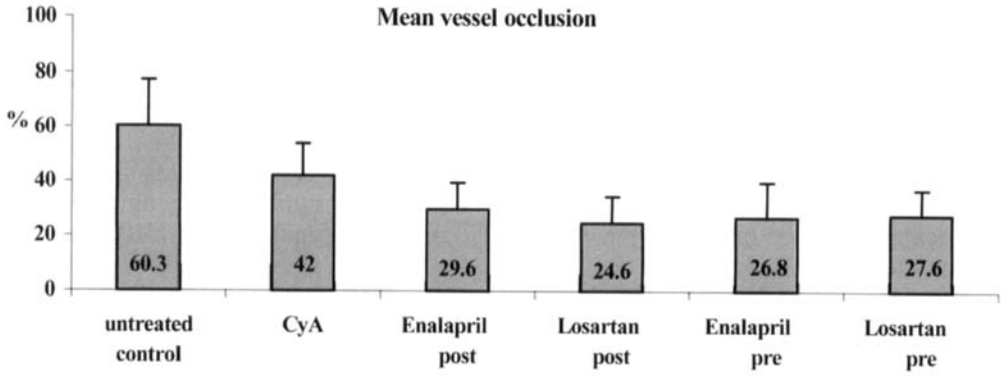

Fig. 3 Mean vessel occlusion

Pre-treatment with enalapril and losartan, maintained and combined with CyA immediately after transplantation, decreased the incidence to $62.1 \pm 11.1\%$ and $66.5 \pm 9.4\%$, respectively, which were both not significant compared with CyA, but significant in comparison with untreated control animals. Only losartan, when started immediately after cardiac transplantation (post-treatment) in combination with CyA, reduced the incidence to $54.2 \pm 16.5\%$, which was statistically significant when compared with untreated controls, CyA-treated animals ($P < 0.05$), and enalapril post-treated animals ($P < 0.05$).

Mean vessel occlusion by TVP in coronary arteries

Mean vessel occlusion by TVP in coronary arteries is shown in Fig. 3. Neointimal proliferation in coronary arteries after cardiac transplantation led to an MVO of $60.3 \pm 12\%$ in untreated animals and $42 \pm 12\%$ in CyA-treated animals ($P < 0.05$). Additional treatment with enalapril after transplantation (post-treatment) further reduced the MVO to $29.6 \pm 10\%$, but this did not reach statistical significance when compared with CyA-treated animals ($P = 0.07$). However, pre-treatment with enalapril as well as pre- and post-treatment

with losartan in addition to CyA significantly reduced the MVO caused by neointimal proliferation to 26.8 ± 13%, 27.6 ± 13%, and 24.6 ± 10.8%, respectively ($P < 0.05$ vs CyA alone).

Discussion

CyA-based immunosuppression has resulted in a marked improvement in allograft and patient survival. Survival rates greater than 85% for heart allografts in the 1st year after cardiac transplantation have, however, led to the recognition of a late, serious, and potentially life-threatening complication, the development of TVP [28]. The dominant pathological findings in TVP in cardiac allografts consist of diffuse, concentric myointimal proliferation lesions in both large and medium-sized arteries and arterioles, often extending through the entire length of the vessel wall [18]. TVP may develop as early as 3 months after heart transplantation and is seen in up to 50% of transplant recipients after 3–5 years [30]. There is still no option in therapy strategies to prevent TVP after transplantation, and the incidence of TVP has still not declined under the use of current immunosuppressive regimes.

From experimental data it is suggested that Ang II contributes to the initiation or progression of TVP. First, Ang II has direct proliferative effects on VSMCs by binding to the AT_1 receptor. Ang II stimulates growth, migration, and matrix production in VSMCs. Secondly, it promotes a variety of molecular events responsible for the local up-regulation of the RAS. Ang II induces adhesion molecule expression in endothelial cells [26] and activates monocytes. Ang II by itself is chemotactic for T-lymphocytes [8]. T-lymphocyte trafficking to the graft endothelium during allorecognition enhanced ACE expression and influenced the local production of Ang II [8]. ACE, AT_1, and AT_2 are the dominant molecules in neointimal formation in response to injury [15, 26, 40]. The local neointimal Ang-II formation and interaction with the AT_1 receptors leads to an up-regulation of adhesion molecules that build a chemo-attractant surface for T lymphocytes that, after attachment to the endothelium, lead to an up-regulation of local ACE and adhesion molecules [8].

In our study, ACE inhibition was not as effective in the reduction of experimental TVP as was AT_1 blocker therapy. Post-treatment with enalapril had no significant effect on the incidence of diseased vessels and on MVO. Pre-treatment with ACE inhibitors or AT_1 blocker did not reduce the incidence of diseased vessels, but reduced MVO. Only losartan post-treatment reduced the incidence of diseased vessels in addition to the reduction of MVO when compared with CyA, controls, and even enalapril post-treatment.

The reduction of TVP by additional therapy with ACE inhibitors is a well-investigated phenomenon and has been described in non-transplant intimal hyperplasia and transplant models [10, 20, 23]. ACE, a dipeptidyl carboxypeptidase, converts Ang I into Ang II, but only 20–40% of the total Ang II is converted by ACE [11]. A local source of Ang II production is located in endothelial chymase [38] that cannot be blocked by ACE inhibitors [3]. The main task of ACE is inactivation of bradykinin, which is responsible for the anti-proliferative properties of ACE inhibitors [7]. During ACE inhibition, local elevated bradykinin levels activate the arachnoid-acid metabolism that leads to the production of prostaglandins and nitric oxide (NO) [12], which have anti-proliferative properties on VSMCs [27]. The anti-proliferative effect of ACE inhibitors can be abolished in a response-to-injury model by therapy with the bradykinin blocker HOE-140 or the NO antagonist L-NAME [12]. In human skin fibroblasts, it has been shown that bradykinin-induced arachidonic acid release, prostaglandin production, and increase in cyclic AMP were blocked by the phospholipase inhibitors mepacrine and dexamethasone [12, 19, 27]. Through pre-treatment with enalapril we observed a reduction of MVO in our experiment. Possibly at the time of transplantation the locally influenced RAS is too weak to stimulate VSMC migration and proliferation. The reduction could also be due to up-regulation of circulating Ang II and the consecutive desensibilization of AT_1 receptors as described during chronic ACE therapy [4]. Unfortunately, we could not confirm our hypothesis with immunohistochemical studies. In the case of post-treatment with enalapril, we observed no statistically significant reduction ($P = 0.07$) of MVO, but we could see a trend in reduction when compared with CyA-treated animals. This effect may be due to an inappropriate dose of the applied ACE inhibitor. No data concerning the effects of enalapril on TVP in animal transplant models and non-transplant intimal hyperplasia models are available thus far. However, the dose of enalapril applied in the present study is regarded as equieffective to those of other ACE inhibitors used in a variety of experimental studies [23]. After transplantation, there may have been an imbalance between the rapidly up-regulated RAS and the dose of the ACE inhibitor. The insufficient reduction of TVP could also be explained by the chymase-induced production of Ang II. Maybe ACE inhibitors, therefore, also had no impact on the incidence of diseased vessels. While ACE blockade in animal restenosis models has shown a reductive effect in neointima formation, it failed to prevent restenosis in clinical trials. This failure may be due to low-dose inhibition, but also to ACE-independent Ang-II generation by the enzyme chymase [3].

Interestingly, only AT_1 receptor blockade with losartan in combination with CyA, when applied immediately after cardiac transplantation (post-treated),

reduced both MVO and incidence of diseased vessels. This effect might reflect a superiority of blocking the RAS at the site of its receptors. Pre-treatment with losartan also reduced MVO, but had no effect on the incidence. For this result we have no further explanation.

ACE inhibition after cardiac transplantation can reduce CyA-induced hypertension and restore the leakage of NO production, which might also be responsible for chronic renal failure during CyA therapy [17]. AT_1 receptor blockers have similar antihypertensive and reno-protective effects. ACE and AT_1 inhibitors reduce a variety of growth-promoting factors such as TGF-ö, platelet-derived growth factor (PDGF) A and B, insulin-like growth factor (IGF)-1, and interleukin-1 (IL-1) [37], which is explained by the reduction of the mitogen-activated protein kinase (MAPK) system responsible for proto-oncogene synthesis and synthesis of the activator protein-1 (AP-1) complex necessary for DNA translation [21, 25]. However, ACE inhibitors are unable to block the extra Ang-II production [19], and the beneficial effect on VSMCs is abolished by corticoids [21], which are an essential cornerstone in triple immunosuppression. Therefore, AT_1 receptor blockade after transplantation may be beneficial. Losartan binds insurmountably to the AT_1 receptor and provides a more complete blockade of the negative cardiovascular effects of Ang II than is possible with ACE inhibition. The chronic use of losartan leads to an elevation of Ang II, which can act at other angiotensin bindings sites, for example, the anti-proliferative AT_2 receptors [4], which are re-expressed on neointimal smooth muscle cells (SMCs) [40]. Elevation of circulating Ang-II levels leads to a desensitization and down-regulation of AT_1 receptors [21]. This side effect could also have contributed to the reduction in incidence and severity of TVP in our animal trial. Corticoids increase the expression of AT_1 receptors on SMCs [22] and, therefore, AT_1 blockade after transplantation could be an efficient way to prevent the Ang-II response at the site of the receptor, unaffected by local extra ACE-mediated Ang-II production. Further studies will have to show whether an increase in dosage of applied AT_1 blockers is accompanied by an increase of anti-proliferative properties, but so far we think that therapy with effective blockers of angiotensin receptors, such as losartan, is a selective approach for therapy of TVP and should be confirmed in clinical studies.

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