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ORIGINAL ARTICLE

Late treatment with angiotensin-converting enzyme inhibitors plus endothelin receptor antagonists ameliorates rat tracheal allograft rejection

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Summary

Inhibition of the renin-angiotensin and endothelin (ET) systems prevents the development of obliterative airway disease (OAD) in rat tracheal allografts. In this study, we assessed whether these therapeutic approaches are effective even when the same were started after signs of OAD were already manifest. Rat tracheas were heterotopically transplanted from Brown-Norway donors into Brown-Norway or Lewis recipients. Allograft recipients received bosentan, ramipril, bosentan plus ramipril or vehicle from day 10 to 24. Untreated allografts and isografts were harvested at day 10 or 24. In tracheal grafts, morphometric studies together with molecular analysis by real-time PCR were performed. Fibroproliferative process in untreated tracheal allografts but not in isografts started already at day 10. Neither bosentan nor ramipril treatment alone as monotherapy could modify the development of OAD when administered only between day 10 and day 24. By contrast, the combination treatment of bosentan and ramipril ameliorated airway obstruction by day 24, which was accompanied by reduced mRNA expression of intragraft transforming growth factor-β1 and platelet-derived growth factor-A and -B chains. Only the combined blockade with angiotensin-converting enzyme inhibitors and ET receptor antagonists can reduce the progression of OAD in this model if the treatment is initiated late in the disease course.

Introduction

Bronchiolitis obliterans syndrome (BOS) is a fibroproliferative process that manifests clinically as chronic graft deterioration after lung transplantation [1]. Early histopathological features of BOS include peribronchiolar leukocyte infiltration and disturbance of the respiratory epithelium, which is followed by the proliferation of mesenchymal cells and matrix deposition leading ultimately to airway obliteration.

Angiotensin II, the main effector peptide of the renin-angiotensin system (RAS) has been implicated in numerous fibroproliferative processes besides its hemodynamic effects. Similarly, the vasoconstrictor endothelin (ET)-1 exerts pro-fibrogenic and growth-promoting effects in various tissues. Recently, we have demonstrated that both angiotensin-converting enzyme (ACE) inhibitors and the ET receptor antagonists retard progression of obliterative airways disease (OAD) in the rat tracheal allografts [2], which is an established animal model for study of bronchiolitis obliterans (BO) [3]. We have also showed that the interruption of both pathways provides superior graft protection than the blockade of a single system.

Our previous experimental settings were designed to study the effects of preventive interventions; thus treatments were started immediately after transplantation surgery. However, the question we addressed in this study was whether such interventions are still capable of limit-

ing OAD progression if started late in the course of the disease when signs of airway obliteration are apparent. Therefore, in the same rat model we initiated treatments with the ACE inhibitor ramipril and the dual ET receptor blocker bosentan alone or in combination 10 days after the transplantation. We also investigated the effects of ACE inhibition and ET receptor blockade on mRNA levels of growth factors and chemokines commonly implicated in tracheal allograft rejection.

Materials and methods

Animals and transplantation surgery

Inbred male Brown-Norway (BN; WOBE Laboratories, Budapest, Hungary) and Lewis (Lew) rats weighing 250–300 g were used. Allogenic heterotopic tracheal transplantations were performed from BN to Lew rats, as previously described [4]. Syngenic controls involved transplantation from BN to BN rats. The ischemic time *ex situ* was 2–5 min. To overcome infectious complications, recipients received ceftriaxone (Rocephin, 20 mg/kg i.m.; Roche, Budapest, Hungary) after the operation. Recipients received no immunosuppression. Animals were kept under standard conditions and were fed rat chow and water *ad libitum*. All animals received humane care in compliance with the 'Principles of Laboratory Animals Care' published by the National Institutes of Health (NIH Publication Vol. 25, No. 28 revised 1996).

Experimental groups

Allograft recipients were divided into five experimental groups (n = 8/group). Animals in the first group were not treated and were harvested at day 10 after transplantation. In the remaining four groups, rats were treated either with the dual ET-1 receptor blocker bosentan (B, 100 mg/kg; Actelion Ltd, Allschwil, Switzerland), ACE inhibitor ramipril (R, 5 mg/kg; Aventis Ltd., Budapest, Hungary), bosentan plus ramipril (B + R, doses as noted before) or vehicle (V) from day 10 until harvesting at day 24. Isograft recipients (I) were divided into two groups, had no treatment and were harvested at day 10 or 24 (n = 8/group). All drugs were applied daily by oral gavage (1 ml solute/animal) in doses used in our previous experiment [2].

Histological evaluation

For histology, tracheal grafts were fixed in buffered formalin (4%), embedded in paraffin and then stained with the Mayer's hematoxylin-eosin (HE) solution. Sections were utilized for computerized morphometry performed with a microscope (Olympus CH 30 microscope; Olympus Inc., Budapest, Hungary), an attached video camera (Olympus DP-10) and a morphometric software (Olympus DP-Soft), as previously described [2,4]. In the analysis, the percentages of both the epithelial loss and the luminal obliteration were calculated. All morphometric analysis was performed by two observers in a blinded fashion.

Molecular analysis

Total RNA from the tracheal grafts was extracted with TRI-Reagent (Sigma, Sigma-Aldrich Ltd, Budapest, Hungary) as previously described [5]. cDNA was synthesized with the TaqMan Reverse Transcription Reagents Kit (Applied Biosystems, Applera Ltd, Budapest, Hungary) according to the manufacturer's protocol. The reaction mixture contained: 1x TaqMan RT Buffer, Magnesium Chloride (5.5 mM), adenosine triphosphate, thymidine triphosphate, guanosine triphosphate, and cytosine triphosphate each at a concentration of 500 μM, Oligo$(dT)_{12\text{-}18}$ primer (2.5 μM), RNase inhibitor (0.4 U/μl), MultiScribe Reverse Transcriptase (1.25 U/μl) and 3.85 μl RNA sample volume in 10 μl reaction volume. The reaction was allowed to proceed (incubation: 25 °C, 10 min, RT: 48 °C, 30 min; Perkin-Elmer Thermal Cycler, Model 9600; Perkin-Elmer, Norwalk, CT, USA), and then it was halted by heating the samples to 95 °C for 5 min followed cooling on ice.

Specific cDNA products corresponding to mRNA for transforming growth factor (TGF)-β1, platelet-derived growth factor (PDGF)-A and -B chain and monocyte chemoattractant protein (MCP)-1 were amplified using real-time PCR with the TaqMan Universal PCR Master Mix Kit (Applied Biosystems) according to the manufacturer's protocol. Two microliters RT reaction was taken for each PCR. Primers were obtained from Applied Biosystems. Amplification was performed with the same sequence profile, as previously described [4]. The relative level of mRNA expression of a specific gene was calculated based on ΔΔCT method according to the manufacturer's instruction with software package (BIORAD, Gene Expression Analysis for Real-Time PCR Detection System; BIORAD Hungary Ltd, Budapest, Hungary), and normalized to mRNA level for the housekeeping gene such as glyceraldehydes-3-phosphate dehydrogenase (GAPDH).

Statistical analysis

Results are reported as mean ± standard error of the mean (SEM). Data were compared using analysis of variance (ANOVA) followed by multiple pair-wise comparison according to the Newman–Keuls test. Differences were considered statistically significant at $P < 0.05$.

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Results

Histology

In isografts (I) harvested at day 10 or 24 after transplantation, the tracheal wall structure was well-preserved, and the cross sectional area of the tracheal lumen was narrowed by approximately 5%. As noted previously in this model [3] the normal mucosal and submucosal thickness accounts for this narrowing. As a characteristic of this model, the obliterative process in allografts harvested at day 10 had already started and resulted in 37.4 ± 4.9% obliteration (Fig. 1a). The difference in occlusion noted

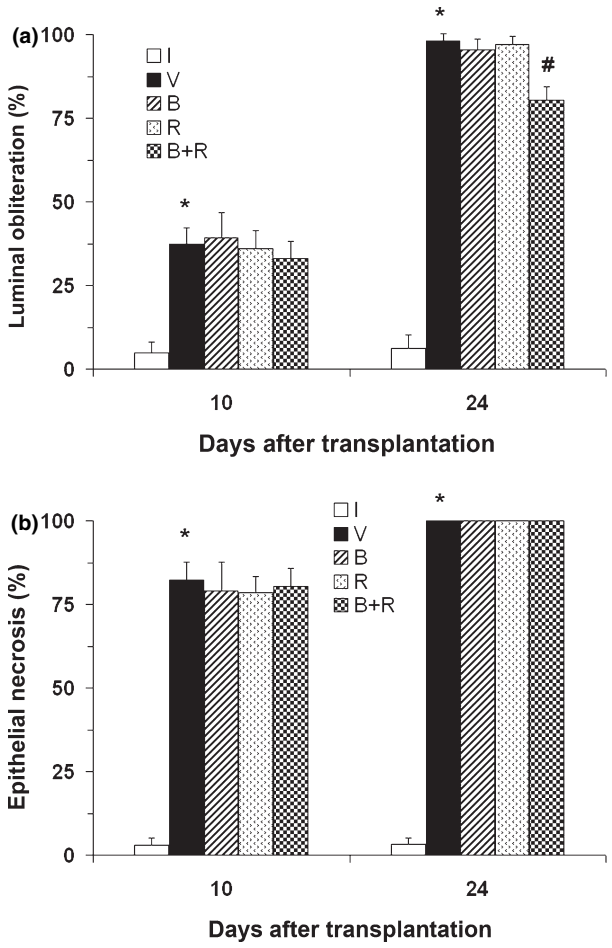

Figure 1 Percentage of luminal obliteration (a) and epithelial necrosis (b) in tracheal iso- and allografts at 10 and 24 days after transplantation (*$P < 0.05$ vs. isograft recipients; #$P < 0.05$ vs. vehicle-treated allograft recipients).

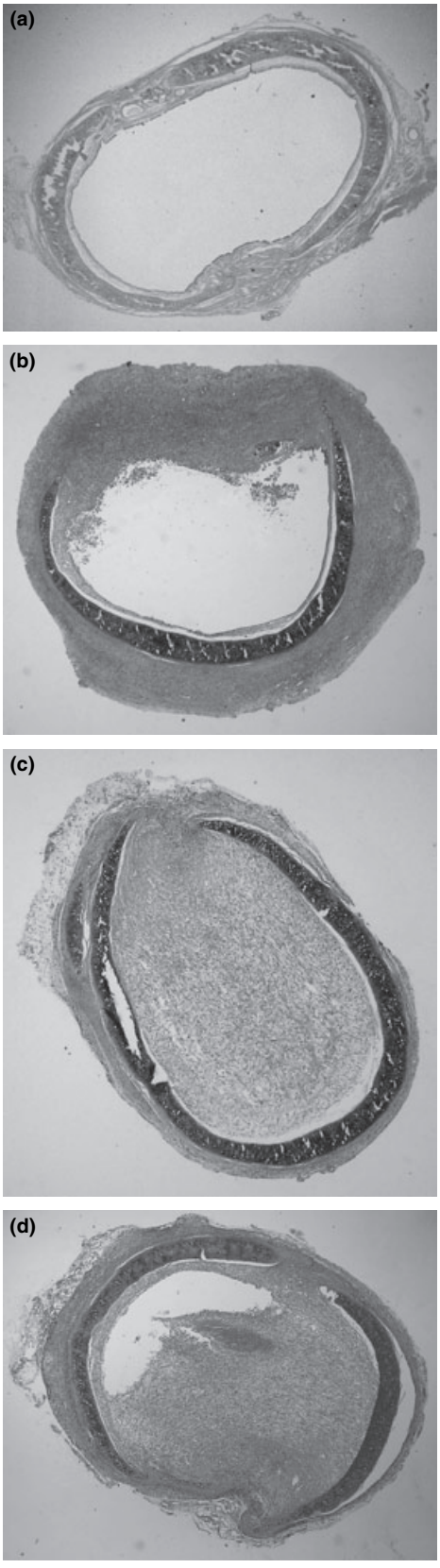

Figure 2 Representative sections of tracheal isografts (a), allografts harvested at day 10 (b), and allografts harvested at day 24 in vehicle- (c) and bosentan plus ramipril-treated animals (d).

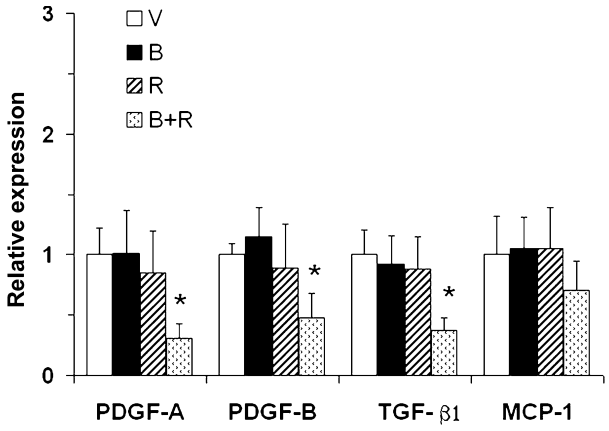

Figure 3 Platelet-derived growth factor (PDGF)-A and -B chain, transforming growth factor (TGF)-β1 and monocyte chemoattractant protein (MCP)-1 mRNA expressions in tracheal allografts at 24 days after transplantation (*$P < 0.05$ vs. vehicle-treated allograft recipients).

between the iso- and allografts was significant ($P < 0.05$). In the vehicle-treated animals (V), airway lumina were completely obliterated by fibroproliferative tissue at day 24 (V: 98.6 ± 3.3%). Similarly, in bosentan- (B) and ramipril-treated (R) animals the percentage of obliteration was almost 100%. By contrast, in animals having received the combined treatment (B + R), significantly lower graft obliteration was observed compared with vehicle-treated rats (B + R: 80.3 ± 4.1%; $P < 0.05$) (Fig. 2).

Isografts (I) examined at day 10 or 24 showed approximately 98% preservation of the respiratory epithelium with normal-appearing ciliated epithelial cells. No inflammatory cell infiltration was noted. By contrast, allografts harvested at day 10 revealed already an 82.2 ± 5.5% loss of the respiratory epithelium with marked inflammatory cell infiltration in the submucosa and the surrounding tissues (Fig. 2). At day 24 in vehicle-treated allografts (V) the epithelial necrosis was complete. None of the treatments (B, R, B + R) reduced epithelial necrosis at this time point (Fig. 1b).

Molecular analysis by real-time PCR

Intragraft mRNA expressions for growth factors including TGF-β1, PDGF-A and -B chain were significantly increased in allograft tracheas compared to isografts at day 24 ($P < 0.05$) (data not shown). Similarly, MCP-1 mRNA levels were up-regulated in allograft tracheas ($P < 0.05$). Neither bosentan (B) nor ramipril (R) monotherapy influenced these levels by the end of the study (Fig. 3). By contrast, rats having received the combined treatment (B + R) showed reduced intragraft TGF-β1, PDGF-A and -B chain levels ($P < 0.05$). MCP-1 mRNA expression was also reduced in these rats; however, this difference was not statistically significant ($P > 0.05$).

Discussion

This study demonstrates that late treatment with ACE inhibitors or ET-1 receptor antagonists alone cannot prevent the development of OAD in the rat tracheal allograft model whereas the dual blockade of the RAS and ET systems ameliorates fibrous airway obliteration even if combination treatment is initiated when signs of OAD are already manifest. Beneficial effects of the combined treatment were associated with decreased growth factor mRNA expressions.

It is generally accepted that both angiotensin II and ET-1 exert various nonhemodynamic actions such as promoting cellular proliferation and growth, stimulating extracellular matrix protein synthesis and regulating inflammatory processes. ET-1 has been implicated in the pathogenesis of several lung diseases including pulmonary vascular disease, pulmonary fibrosis and asthma [6]. Moreover, ET-1 might be an important mediator in pathological processes related to lung transplantation such as ischemia/reperfusion injury [7] or the development of BOS in experimental settings [8]. Similarly, the involvement of angiotensin II in the fibroproliferative response to lung injury is well recognized [9]. Several lines of evidence suggest that both the RAS and the ET systems are activated in a rat model of BO, and results of our [2] and other [10,11] groups demonstrated that RAS inhibitors or ET receptor antagonists can prevent the development of OAD in this model.

All these interventional studies were in fact 'preventive' in terms of their design, i.e. treatment regimens were applied prior to the manifestation of morphological signs of OAD. In this study, the first set of allograft recipients was harvested at day 10, which is beyond the peak (day 7) of the so-called lymphocytic phase of this model and it is at the beginning of the obliterative phase [12]. These allografts already showed modest luminal obliteration. Thus, we initiated our treatments at this time point, and demonstrated for the first time that the combination treatment with ACE inhibitors and ET receptor blockers attenuated disease progression, while the respective monotherapies had no protective effect. These data are in agreement with our previous findings showing a therapeutic benefit of dual-agent compared to single-agent blockade when treatment is started immediately after transplantation [2].

The lack of therapeutic effect in groups under monotherapy may probably be explained by the relatively late harvesting time point (day 24) in our experimental protocol. It is possible that at an earlier time point, we might

have seen some protective effect in bosentan- and ramipril-treated rats. Alternatively, beyond a certain time point in this model, lesion development would irreversibly progress despite the fact that a single pathway is blocked.

It is of note that even the combined treatment had no beneficial effect on epithelial damage. In general, this is in line with previous findings utilizing ACE-inhibitors or ET receptor blockers in the same model [10,11]. It is possible that the blockade of the RAS or ET system is simply ineffective in preventing alloimmune-mediated epithelial injury in this setting.

Few reports have studied the effects of late inhibition of either the RAS or ET system on graft injury in transplanted organs. Using the Fisher-to-Lewis rat kidney model, Noris *et al.* [13] recently demonstrated that the ACE inhibitor trandolapril limits chronic allograft nephropathy even in case of treatment that was started when onset of impaired graft function and glomerulosclerosis has already been noted. In the same model, late treatment with angiotensin type 1 receptor blockers also attenuates renal allograft injury [14]. Nevertheless, in these settings no combination therapy with ET receptor antagonists was applied.

Several lines of evidence indicate that the contribution of angiotensin II and ET-1 to tissue remodeling is linked to the autocrine release of the key multifunctional cytokine TGF-β1. TGF-β1 exerts strong pro-fibrogenic activities in many organs by promoting matrix protein synthesis and inhibiting its degradation. In lung fibroblasts both angiotensin II and ET-1 induce collagen production via the autocrine action of TGF-β1 [15,16]. Angiotensin II-mediated cellular hypertrophy in bronchial smooth muscle cells is also induced by TGF-β1 [17]. In transplantation, TGF-β1 is considered to be a central mediator of the fibroproliferative process associated with BOS, both in experimental models [18] and humans [19]. In our study, rats treated with bosentan plus ramipril demonstrated decreased mRNA expression of TGF-β1, which may in part be responsible for the attenuated airway obliteration in these allografts.

Another growth factor, which has been generally implicated in the pathogenesis of BOS is PDGF. It is a disulfide-bonded dimer of two subunit polypeptides named PDGF-A and -B chains. Apart from being strongly mitogenic for smooth muscle cells and fibroblasts, PDGF stimulates extracellular matrix production in the lung [20]. Lung transplant patients with BOS have elevated PDGF levels of bronchoalveolar lavage fluid [21]. Furthermore, it has been showed that blocking PDGF receptors reduces allograft rejection in a rat model of BO [22]. In our study PDGF mRNA expressions, both of A and B chains, were significantly reduced in rats having received combined treatment, which might have contributed to reduced allograft damage in these animals. By contrast, in groups with monotherapy no effect on PDGF and TGF-β1 mRNA levels was noted, which is in line with the histological findings.

There is evidence that angiotensin II may enhance lymphocyte- and monocyte recruitment in many organs by inducing chemokines such as MCP-1, which is one of the most frequently studied chemoattractant for monocytes/macrophages [23,24]. RAS inhibitors, on the other hand, decrease MCP-1 levels, and this may contribute to their organ-protective effects in many experimental and human settings. The relationship between ET-1 and MCP-1 has been less extensively studied; however, for example, ET-1 may increase MCP-1 levels in glomerular mesangial cells [25].

The contribution of MCP-1 to inflammatory cell recruitment in BOS is well recognized. Lavage fluid of patients with BOS shows elevated levels of this chemokine [26,27], and inhibiting MCP-1 seems to limit the progression of BO, at least in experimental conditions [26,28]. In our study MCP-1 mRNA levels were elevated in allografts compared to isografts at day 24, supporting the results of previous studies in the rat [29] and murine [26] models. In rats treated with the combination therapy, MCP-1 levels were slightly decreased, which may again contribute to less severe allograft injury in these rats.

In our study, we have used the well-established heterotopic tracheal transplant model in which reproducible occlusion of the airway lumens occurs in allografts. The development of this lesion is pathologically similar to what has been observed in human BO, and therefore serves as a viable, convenient and technically less demanding alternative to orthotopic whole lung transplant models in this species.

At present, it is not known whether ACE inhibitors and/or ET-1 receptor antagonists can slow down the progression of BOS in humans. Similarly, it is unclear what would be the optimal time-point of initiation of such potentially beneficial treatment. Although we acknowledge the limitations of this rat model, our findings may have important clinical implications. First, our data suggest that potentially favorable drugs such as bosentan and ramipril may lose their protective capacity after a certain point in disease course. Second, even in the late phase of BOS, the combination of these agents might bring some clinical benefit.

In conclusion, we demonstrate that the dual blockade of the RAS and ET systems can reduce the progression of OAD in rat tracheal allografts even if treatment is started late in the course of disease, when lesions have already set in. Translating these observations into clinical practice, it seems that different therapeutic strategies should be applied at different disease stages in order to enhance

graft longevity and reduce the progression of chronic allograft injury in patients with BOS.

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Authorship

BA: designed the study, performed experimental work, collected and analyzed data, wrote the paper. JF: performed histology, analyzed data. AS: performed real-time PCR, analyzed data. CJ: performed real-time PCR, analyzed data. IH: designed research, corrected the paper.

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