Original Article

Influence of growth factors on the proliferation of vascular smooth muscle cells isolated from subtotally nephrectomized rats after endothelin or angiotensin II antagonism

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Abstract

Background. Cardiovascular disease is the most important cause of death in patients with end-stage renal disease. In uraemia, the renin–angiotensin–aldosterone and endothelin (ET) systems are activated. It is not known whether inhibition of these systems attenuates the proliferation of isolated smooth muscle cells of uraemic rats.

Methods. Subtotally nephrectomized (SNX) rats were treated with an ETA receptor antagonist, an ETAB receptor antagonist, the angiotensin type 1 (AT1) receptor antagonist losartan (all 10 mg/kg body weight/day) or the angiotensin-converting enzyme (ACE) inhibitor trandolapril (0.1 mg/kg body weight/day) or received no medication (SNX) for 12 weeks. Then, aortal smooth muscle cells (SMCs) were isolated and cultivated. After incubation of SMCs with different growth factors (5–7 days), proliferation was measured using a bromodeoxyuridine enzyme-linked immunosorbent assay (BrdU ELISA).

Results. Higher maximum levels of proliferation were found in SMCs from untreated SNX rats than in SMCs from control animals [platelet-derived growth factor-BB (PDGF-BB) 486.60 ± 8.27 vs 346.74 ± 4.60%, basic fibroblast growth factor (bFGF) 176.68 ± 6.50 vs 123.71 ± 1.49%, tumour necrosis factor-α (TNF-α) 153.38 ± 10.16 vs 122.27 ± 1.41%]. Treatment with ET receptor antagonists or losartan reduced growth factor-induced SMC proliferation in vitro. However, further investigations with uraemic patients have to clarify whether angiotensin or ET receptor antagonists inhibit the development of atherosclerosis.

Conclusions. Treatment of SNX rats with ET receptor antagonists or losartan reduced growth factor-induced SMC proliferation in vitro. However, further investigations with uraemic patients have to clarify whether angiotensin or ET receptor antagonists inhibit the development of atherosclerosis.

Keywords: growth factors; proliferation; SMC; uraemia

Introduction

Patients with end-stage renal disease (ESRD) have a significantly higher risk of suffering from cardiovascular disease (CVD) than the general population. Even though several traditional risk factors for atherosclerosis are associated with ESRD, they cannot fully explain the fact that CVD mortality is 10–20 times higher in uraemic patients [1]. Certain specific additional risk factors such as increased oxidative stress, uraemic toxins and a chronic inflammatory state with an increase in pro-inflammatory cytokine and growth factor levels are thought to contribute to the development and progression of atherosclerosis in uraemia. Furthermore, the activation of the renin–angiotensin–aldosterone system (RAAS) and the endothelin (ET)-1 system [2] seems to play a role. Therefore, inhibiting these systems with angiotensin-converting enzyme (ACE) inhibitors, angiotensin type 1 (AT1) receptor antagonists or ET receptor antagonists may represent promising strategies to prevent the accelerated development of atherosclerosis in uraemia.
During the past several years, experimental data have illuminated the role of inflammation in atherogenesis [3] with the recruitment of macrophages and T lymphocytes to the developing lesion, where they secrete numerous growth factors. Thereby, they are able to stimulate proliferation and migration of smooth muscle cells (SMCs).

In uraemia, pro-inflammatory cytokine and growth factor levels [e.g. tumour necrosis factor (TNF-α), interleukin (IL)-1, IL-6, C-reactive protein (CRP), monocyte chemotactic protein-1 (MCP-1)] are increased [4]. It is not known, however, whether SMCs in uraemia respond differently to growth factors. Moreover, the effect of ET-1 or angiotensin II antagonism on SMC proliferation rate has not been clarified to date.

In the present study, we investigated whether the proliferative response to several growth factors differs between aortal SMCs isolated from control and uraemic rats and how treatment of uraemic rats with different antihypertensive drugs influences the response to these growth factors.

**Methods**

**Animals**

Male Sprague–Dawley rats weighing ~200 g were purchased from Charles River, Kisslegg, Germany, and housed individually. All except for the control animals underwent a two-step five-sixths subtotal nephrectomy (SNX). First, the right kidney was removed under anaesthesia with ketamin (50–15 mg/kg body weight) and xylazin (5–15 mg/kg body weight). One week later, the lower and upper poles of the remaining kidney were dissected. After surgery, one group of animals received no treatment (SNX), the others were treated with different antihypertensive drugs (10 mg/kg body weight/day) or losartan (MSD Sharp and Dohme, Haar, Germany; 10 mg/kg body weight/day), trandolapril (Knoll; 0.1 mg/kg body weight/day), the unselective ETα receptor antagonist LU 302872 (Knoll, Ludwigshean, Germany; 10 mg/kg body weight/day), AT1a receptor forward, GCCAGTTTGCCAGCTGT CAT; AT1a receptor reverse, CGCGCACACTGTGATAT CAT; AT2 receptor reverse, TGATCCCCACAGAAGCC TTC; AT1a receptor forward, GCCAGTTTGCGAGCTC TGG; ATB receptor forward, TGGAGGCTGAGTGTGCA AGC; ETα receptor forward, ATTGCCCTACGGAA CAC; ETα receptor reverse, CAAAACAGCAAGAAGGCC GTC; ETB receptor forward, TGGGCTGAGATGTGCA AGC; ETB receptor reverse, TGATCCCCACAGAAGGCC TTC; AT1a receptor forward, GCCAGATTGGCGAGCT C TGG; ATB receptor forward, ATCCCTGTGAAAGCAT TATG; AT2 receptor reverse, ATGGTGGAATGAGGA CAGACA. Polymerase chain reaction (PCR) amplification was performed in triplicate (2 min at 50°C, 10 min at 95°C, 15 s at 95°C and 1 min at 60°C for a total of 40 cycles) in an ABI Prism™ 7700 sequence detector (Applied Biosystems). The expression of treated (sample) cells relative to control and SNX animals were conducted with unpaired t-test following by Dunnett’s test. Bonferroni’s multiple comparison test was used to determine the differences in proliferation between SMCs from different animals under the influence of one growth factor concentration. Statistical analyses of ET and AT receptor mRNA expression in control and SNX animals were conducted with unpaired t-test.

**Cell culture**

Primary cultures of SMCs were obtained by isolating the cells growing out from small pieces of the explanted aortas cultivated in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 20% fetal calf serum (FCS; BioWhittaker) and 5% penicillin (100 U/ml)/streptomycin (100 μg/ml; Gibco) using cloning rings. A few days later, cell clones were isolated. Smooth muscle origin was confirmed immunocytochemically using a monoclonal antibody against smooth muscle α-actin (Sigma-Aldrich).

**Statistical analysis**

The effects of different growth factor concentrations on SMC proliferation were analysed using one-way ANOVA, followed by Dunnett’s test. Bonferroni’s multiple comparison test was used to determine the differences in proliferation between SMCs from different animals under the influence of one growth factor concentration. Statistical analyses of ET and AT receptor mRNA expression in control and SNX animals were conducted with unpaired t-test.
or Dunnett’s test, respectively. P-values ≤0.05 were considered statistically significant. All data are presented as a percentage of the respective controls. The mean ± SEM of at least three replicates was used for statistical comparison.

**Results**

**Decrease of myocardial angiotensin and endothelin receptor mRNA expression in uraemic rats**

Real-time RT–PCR revealed a significant downregulation of ET<sub>A</sub> and ET<sub>B</sub> receptor mRNA expression in the left ventricle of SNX rats (ET<sub>A</sub> receptor, 41.11 ± 1.59%, n = 3, P ≤ 0.01 vs control, 100 ± 11.01%, n = 3; ET<sub>B</sub> receptor, 23.7 ± 12.85%, n = 3, P ≤ 0.05 vs control, 100 ± 13.83%, n = 3; data not shown). The analysis of AT<sub>1a</sub> and AT<sub>2</sub> receptor mRNA expression revealed a dramatic reduction to undetectable levels in SNX rats.

**Decrease of angiotensin and endothelin receptor mRNA expression in cultured SMCs from SNX rats**

In cultured SMCs from SNX rats, downregulation of AT<sub>1a</sub>, AT<sub>2</sub>, ET<sub>A</sub> and ET<sub>B</sub> receptor mRNA was determined by real-time RT–PCR (AT<sub>1a</sub> receptor: SNX 74.5 ± 5.45%, n = 9, P ≤ 0.05 vs control, 100 ± 3.24%, n = 12; trandolapril 42.4 ± 3.77%, n = 6, P ≤ 0.05 vs control, 100 ± 10.21%, n = 9; trandolapril 183.4 ± 44.55%, P ≤ 0.05; losartan 110.7 ± 12.84%, n = 6, P ≤ 0.01; ET<sub>A</sub> antagonist 145 ± 21.24%, n = 3, P ≤ 0.01; ET<sub>AB</sub> antagonist 39.2 ± 2.47%, n = 3, P ≤ 0.05; AT<sub>2</sub> receptor: SNX 28.1 ± 3.14%, n = 6, P ≤ 0.05 vs control, 100 ± 12.34%, n = 6; P ≤ 0.05; losartan 183.4 ± 44.55%, n = 6, P ≤ 0.05; AT<sub>1a</sub> antagonist 806.6 ± 139.6%, n = 3, P ≤ 0.01; ET<sub>A</sub> receptor: SNX 166.2 ± 50.6%, n = 6, P ≤ 0.05; AT<sub>AB</sub> antagonist 1040.0 ± 105.0%, n = 4, P ≤ 0.01; ET<sub>A</sub> antagonist 166.2 ± 50.6%, n = 6, P ≤ 0.05 vs control, 100 ± 7.91%, n = 9; ET<sub>B</sub> receptor: SNX 57.8 ± 3.37%, n = 6, P ≤ 0.01 vs control, 100 ± 13.83%, n = 3; data not shown).

**Differences between control and SNX animals in terms of SMC proliferation**

To study the effects of uraemia *in vivo* on the proliferation of isolated SMCs, the response of cultured SMCs from uraemic and control animals to PDGF–BB, bFGF, TNF-α, angiotensin II, aldosterone and ET-1 was determined.

Low concentrations of PDGF–BB (10⁻¹³–10⁻¹₂ mol/l), bFGF (10⁻¹³–10⁻¹² mol/l) and TNF-α (10⁻¹³–10⁻¹⁰ mol/l) increased growth in SMCs derived from the control animals (P ≤ 0.05 compared with cells treated with cytokine-free medium). SMCs derived from SNX animals showed no increase in proliferation when incubated with these concentrations. After incubation with high concentrations of the same growth factors, SMCs from SNX rats showed a higher increase in proliferation than control SMCs. TNF-α (10⁻⁹ mol/l) increased proliferation to 153.38 ± 10.16% vs 122.27 ± 1.41% in control (n = 8, P ≤ 0.01, Figure 1a), 10⁻⁹ mol/l PDGF–BB resulted in 486.60 ± 8.27% vs 346.74 ± 4.60% in control (n = 8, P ≤ 0.01, Figure 1b) and 10⁻¹⁰ mol/l bFGF stimulated proliferation to 176.68 ± 6.50% (n = 6) vs 123.71 ± 1.49% (n = 4) in control (P ≤ 0.01, Figure 1c).

Angiotensin II and aldosterone stimulated proliferation in control SMCs (10⁻⁷ mol/l angiotensin II, 141.29 ± 3.37%, n = 8; 10⁻¹¹ mol/l aldosterone, 147.01 ± 4.77%, n = 4; P ≤ 0.05 compared with medium control, Figure 1d), whereas they had no proliferative effect on SNX cells (angiotensin II, 98.02 ± 3.02%; aldosterone, 85.62 ± 2.54%; Figure 1e). ET-1 had no effect on the proliferation of control SMCs (maximum 102.36 ± 2.99%, n = 4 vs 100 ± 2.21%, n = 8 in unstimulated cells, P > 0.05) but inhibited growth in SNX SMCs (minimum 81.08 ± 0.26%, n = 4 vs 100 ± 4.46%, n = 8 in unstimulated cells P ≤ 0.01; Figure 1f).

**Influence of treatment with ET receptor antagonists, losartan or trandolapril on SMC proliferation in comparison with untreated SNX rats**

In SMCs from untreated SNX rats, PDGF–BB (10⁻⁹ mol/l) induced the maximum proliferation of 486.60 ± 8.27% (as compared with unstimulated cells, n = 8, P ≤ 0.01). The increase in proliferation induced by PDGF–BB (10⁻⁹ mol/l) was significantly reduced in SMCs from SNX rats treated with the ET<sub>A</sub> receptor antagonist (135.71 ± 1.08%, n = 8, P ≤ 0.01), the ET<sub>AB</sub> receptor antagonist (122.72 ± 0.58%, n = 8, P ≤ 0.01) or the AT<sub>1</sub> receptor antagonist losartan (103.69 ± 1.83%, n = 8, P ≤ 0.01, Figure 2a).

Stimulation with bFGF (10⁻¹⁰ mol/l) was followed by an increase in proliferation to a maximum of 176.68 ± 6.50% (n = 6, P ≤ 0.01) in SMCs from untreated SNX animals. After treatment with the ET<sub>A</sub> receptor antagonist, bFGF-induced SMC growth was reduced to 108.64 ± 1.09% (n = 4, P ≤ 0.01). Blockade of ET<sub>A</sub> and ET<sub>B</sub> receptors reduced the maximum response to 108.2 ± 1.21% (n = 4, P ≤ 0.01). After chronic treatment with losartan, the growth rate was only 121.80 ± 1.19% (n = 4, P ≤ 0.01; Figure 2b).

TNF-α (10⁻⁹ mol/l) stimulated proliferation in SMCs from SNX animals to a maximum of 153.4 ± 10.16% (n = 8, P ≤ 0.01 compared with untreated cells). ET<sub>A</sub> receptor antagonism reduced the maximum response to 115.63 ± 0.41% (n = 8, P ≤ 0.01). ET<sub>AB</sub> receptor antagonism resulted in a higher SMC responsiveness to TNF-α (maximum 202 ± 1.28%, n = 8, P ≤ 0.01). In contrast, after losartan treatment, TNF-α reduced SMC proliferation (minimum 77.82 ± 1.91%, n = 8, P ≤ 0.01, Figure 2c).

SMCs isolated after trandolapril treatment showed a greater increase in proliferation in response to PDGF–BB (10⁻⁹ mol/l), bFGF (10⁻¹⁰ mol/l) and TNF-α (10⁻⁹ mol/l) than cells originating from all other animals. The maximum response to PDGF–BB (10⁻⁹ mol/l) was 663.48 ± 7.00% (n = 8, P ≤ 0.01 compared with SNX) (Figure 2d). bFGF-induced BrdU incorporation reached its peak at 568.81 ± 17.94% (n = 4, P ≤ 0.01, Figure 2e). TNF-α stimulated SMC
proliferation up to 356.34±10.43% \((n = 8, \ p \leq 0.01, \ \text{Figure 2f})\).

**Discussion**

In the present *in vitro* study, we have observed that SMCs isolated from SNX rats show a higher maximum proliferation rate in response to the growth factors PDGF-BB, bFGF, and TNF-α. After treatment with ET \(_A\) and ET \(_{AB}\) receptor antagonists or the AT\(_1\) receptor antagonist losartan, this effect was attenuated. In contrast, the maximum growth factor-induced proliferation in SMCs from SNX rats treated with the ACE inhibitor trandolapril was higher than in SMCs from SNX and control rats. In SMCs from control rats, angiotensin II and aldosterone stimulated proliferation, but did not do so in SMCs from SNX rats.
ET-1 inhibited SMC proliferation in SNX rats but had no effect on control cells. The phenomenon that plasma cytokine levels are increased in uraemia could explain the lack of effect of low PDGF-BB, bFGF and TNF-α concentrations on the proliferation of SMCs isolated from untreated SNX rats in our study. High circulating or local levels of these growth factors in vivo might have resulted in a downregulation of the corresponding growth factor receptors. However, when high concentrations were used, the effect of PDGF-BB, bFGF and TNF-α on proliferation was more pronounced in SMCs derived from the uraemic animals than in controls. This indicates that the uraemic state in vivo could have induced genetic changes in the SMCs still present after isolation and cultivation. This concept is supported further by the different effects that angiotensin II, aldosterone and ET-1 had on proliferation in SMCs from control and SNX rats.
Angiotensin II and aldosterone induced a significant increase in control SMCs, but did not stimulate SMCs from SNX rats. ET-1 inhibited proliferation in SMCs from SNX animals, but had no effect on control cells. In a study on septic rats by Bucher et al. [6], it could be demonstrated that increased angiotensin II plasma levels led to the downregulation of AT$_1$ receptors. Via AT$_1$ receptors, angiotensin II stimulates DNA and protein synthesis in SMCs [7], and enhances the effects of other growth factors [8]. The decrease in angiotensin receptor mRNA expression we found in the myocardium and SMCs of SNX rats can be seen as a downregulation due to an activated RAAS in uraemia in general. A therapy with trandolapril or losartan and ET$_B$ antagonism; both inhibited growth factor-promoted SMC proliferation whereas a mild increase in AT$_2$ receptor gene transcription was not able to reduce the accelerated SMC growth rate.

In the present study, we found SMCs from SNX rats treated with the ETA receptor antagonist to show almost control levels of proliferation in response to PDGF-BB, bFGF and TNF-$\alpha$. This implies that via the ETA receptor, ET-1 might play a role in a certain sensitization of SMCs in vivo, making them more responsive to pro-atherogenic growth factors. SMCs from rats treated with the combined ET$_{AB}$ receptor antagonist also showed control-like reactions to PDGF-BB and bFGF. When stimulated with TNF-$\alpha$, however, their proliferation was stronger than in SNX. This underlines the antiproliferative significance of the ET$_B$ receptor found by Murakoshi et al. [9] and shows the importance of a physiological balance between ETA and ET$_B$ receptors. SMCs from rats treated with the ACE inhibitor trandolapril exhibited a stronger growth factor-stimulated proliferation than SMCs from any other animal. Treatment with trandolapril thus seemed to have sensitized the cells to PDGF-BB, bFGF and TNF-$\alpha$. This might arise from an increase in bradykinin synthesis under trandolapril leading to a sensitization of mitogen-activated protein kinases [10], which is essential for growth factor-mediated stimulation of DNA synthesis [11]. Accordingly, the augmented SMC proliferation in response to PDGF-BB, bFGF and TNF-$\alpha$ seen after trandolapril treatment might be due to a modified state of activation in these cells.

In contrast to our findings, several in vitro studies, and also in vivo studies using balloon angioplasty, demonstrated a growth-inhibiting function of both ACE inhibitors and AT$_1$ receptor antagonists [12]. However, several observations indicated that ACE inhibitors do not always reduce proliferation rates in smooth muscle cells. In a direct in vivo comparison of an AT$_1$ receptor antagonist and the ACE inhibitor temocapril, Teng et al. [13] found that temocapril was not able to inhibit SMC proliferation in spontaneously hypertensive rats (SHRs) whereas it did in Wistar rats. Richter et al. showed in an in vivo model of transplant vasculopathy that the ACE inhibitor enalapril was able to reduce SMC proliferation in small intramyocardial arteries, but not in large epicardial vessels if given before intervention [14]. For an AT$_1$ receptor antagonist, the same group could not demonstrate an antiproliferative function in large epicardial vessels. In our experiments, trandolapril even increased SMC proliferation under different growth factors. Furthermore, Wilson et al. [15] could demonstrate a comparable effect with an increased vessel stenosis using captopril in a model of neointima proliferation in a porcine coronary artery culture model. In these experiments, losartan inhibited neointima formation whereas captopril increased vessel stenosis by 200%. In an experiment with SHRs, Bravo et al. [16] found the proliferation of isolated carotid SMCs to be reduced after 16 weeks of losartan treatment but not after captopril medication. Additionally, cilazapril was demonstrated to inhibit neointimal formation in balloonned guinea pig carotid but not rabbit iliac arteries [17]. In porcine models of restenosis involving injury to the coronary arteries, neither cilazapril [18], enalapril [19], trandolapril nor captopril [20] were effective. Even worse were the results in patients with the ACE gene polymorphism DD, because when these patients were treated with an ACE inhibitor, an increased frequency of in-stent restenosis was found. This indicates that the growth-inhibitory action of ACE inhibitors depends on the genetic background of the individuals and also on the treatment regime [21]. In comparison with these in vivo analyses, our studies were done in vitro after isolation of aortal SMCs.

Limitations of the study

The evolution of atherosclerosis is a very complex mechanism involving SMCs, but also many other components. Rupture of atherosclerotic plaques has been identified as the proximate event in the majority of cases of acute ischaemic syndromes. Vulnerable plaques are characterized by a high lipid content, increased numbers of inflammatory cells and extensive adventitial and intimal neovascularity. The fibrous cap of an atherosclerotic plaque may become thin and rupture as a result of the depletion of matrix components through the activation of enzymes secreted by SMCs. This indicates that SMC proliferation represents only one component of atherosclerosis. Whether the inhibition of SMC proliferation in uraemia reduces the induction and progression of atherosclerosis cannot be fully answered by our investigations. However, in advanced coronary atherosclerotic plaques associated with unstable angina, an augmentation of inflammatory cell activity with significantly increased SMC areas has been shown. Therefore, the inhibition of SMC proliferation might represent one means of reducing susceptibility to plaque rupture.

In summary, we demonstrated that uraemia in the rat entails modifications in SMC responsiveness to
various pro-atherogenic growth factors. These changes are still present when SMCs are isolated and subcultured. Consequently, certain factors present in the uraemic vasculature seem to induce permanent genetic changes in SMCs. Furthermore, we revealed that treatment of SNX rats with ET receptor antagonists or the AT₁ receptor antagonist losartan more or less reduces accelerated proliferation of SMCs. Furthermore, we revealed that changes in SMCs. Furthermore, we revealed that mechanisms involved in the uraemia-related SMC modifications observed in this study should help to come to a deeper understanding of atherogenesis in uraemia.

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