AT1 Receptor Antagonist Candesartan Attenuates Genomic Damage in Peripheral Blood Lymphocytes of Patients on Maintenance Hemodialysis Treatment

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Key Words

Genomic damage • Micronuclei • Angiotensin II •
AT1 antagonists

Abstract

Background: Angiotensin II (ANG II) and advanced glycation end products (AGEs) exert genotoxic effects in vitro which were prevented by the ANG II type 1 (AT1) receptor blocker, candesartan. In end-stage renal disease (ESRD) the incidence of genomic damage is increased. A stimulation of the renin-angiotensin system and accumulation of AGEs could be involved. **Methods:** We tested whether oral co-administration of candesartan modulates enhanced DNA damage in ESRD patients. Fifteen maintenance hemodialysis (MHD) patients with mild hypertension were treated with candesartan for 4.5 months. Fourteen MHD patients served as conventionally treated uremic controls. DNA damage was measured as micronucleus frequency (MNF) in peripheral blood lymphocytes and evaluated three times before candesartan therapy and afterwards every 6 weeks. **Results:** Compared to 14 healthy controls, MNF at baseline was significantly elevated in MHD patients. While in the conventionally treated MHD patients the enhanced DNA damage persisted, the co-administration of candesartan ameliorated the genomic damage significantly and independently of blood pressure

changes. **Conclusion:** Blockade of AT1 receptors with candesartan can reduce DNA damage in MHD patients. Long-term studies in larger patient groups are needed to investigate whether the improved genomic damage lowers atherosclerotic complications and cancer development.

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Introduction

In end-stage renal disease (ESRD) genomic damage is enhanced, as shown in peripheral blood lymphocytes (PBLs) by the micronucleus (MN) frequency (MNF) test and the single cell gel electrophoresis-comet assay (CA) [1–3], elevated 8-hydroxy-deoxyguanosine concentrations in white blood cells [4], sister chromatid exchanges, chromosomal aberrations [5, 6] and mitochondrial DNA deletions [7, 8]. Unrepaired or improperly repaired DNA lesions may have serious consequences, such as premature ageing [9, 10], atherosclerosis [11, 12] and cancer predisposition [13, 14]. Recently, cohort studies have shown a correlation between the frequency of structural chromosomal aberrations or micronuclei in PBLs and an en-

N.S. and P.R. contributed equally to this study.

Table 1. Baseline characteristics of the MHD patients, with and without candesartan treatment

	Candesartan-treated MHD (n = 15)	Conventionally treated MHD (n = 14)
Age, years	63.8 ± 14.6	68.5 ± 10.1
Range, years	29–83	52–82
Male/female	10/5	7/7
Time on MHD, years	6.2 ± 4.1	7.7 ± 5.8
Systolic BP, mm Hg	148 ± 9	136 ± 24
Diastolic BP, mm Hg	81 ± 11	71 ± 15
<i>Cause of end-stage renal disease</i>		
Glomerulonephritis	7	6
Hypertensive nephropathy	4	3
Pyelonephritis	1	
Diabetic nephropathy	1	3
Polycystic kidney disease	0	1
Unknown	2	1
<i>Antihypertensive drug treatment</i>		
β-Blocker	8	10
Calcium channel antagonists	5	3
Central antihypertensive	3	2
Loop diuretics	5	5

hanced cancer occurrence [15, 16]. In patients with coronary artery disease, a positive relationship between micronuclei levels and the severity of the atherosclerotic lesions was found [17]. In ESRD patients, the incidence of cancer [18, 19] and cardiovascular disease [20] is tremendously increased, and the presence of genomic damage in these patients could play a decisive role in the development of these disturbances.

Among the mechanisms of DNA damage and a depressed DNA repair in ESRD [21], numerous factors could be involved, such as formation of reactive oxygen species in the presence of a reduced antioxidative defense mechanism [22] and hypomethylation [23]. Besides the uremic state, the pro-oxidative effect of hemodialysis treatment as well as iron infusion contribute to the observed genotoxicity [24, 25]. The dialysis-induced DNA damage is associated with an enhanced spontaneous DNA repair [26]. Recently, genotoxic effects of various advanced glycation end products (AGEs) as well as of angiotensin II (ANG II) in mammalian cells have been demonstrated, which could be suppressed by both AT1 blockade and antioxidants [27–29]. We hypothesized that the enhanced genomic damage in ESRD patients could be, at least in part, a consequence of an activated AT1 receptor, since the administration of an ACE inhibitor/AT1

receptor blocker has been shown to combat the oxidative stress in hypertensive patients [30–32] and to reduce the morbidity/mortality in maintenance hemodialysis (MHD) patients [33, 34]. To elucidate the potential involvement of AT1 receptors on DNA damage in ESRD patients, we studied the effect of chronic co-administration of the AT1 receptor antagonist, candesartan, in mildly hypertensive patients on MHD therapy. The results obtained demonstrate a significant improvement, suggesting the involvement of AT1 receptors in the pathogenesis of genomic damage in ESRD.

Methods

The study was carried out in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the Medical Faculty of the University of Würzburg. All patients gave their written informed consent prior to participation in this prospective, non-randomized, clinical pilot study.

Subjects

A total of 29 MHD patients (characteristics and antihypertensive treatment are given in table 1) were recruited: 15 stable ESRD mildly hypertensive patients on MHD, without previous treatment with AT1 blockers and/or ACE inhibitors for at least 3 months, were enrolled for the active treatment. The remaining 14 MHD patients served as conventionally treated (i.e. not treated with candesartan) controls, and an additional 14 healthy subjects (7 male/7 female, age 53.4 ± 13.2 years, range 36–79) as controls for the baseline MNF. The age difference between the healthy controls and the treated MHD patients was statistically not significant. Patients with acute or chronic infections as well as carcinomas or congestive heart failure were excluded.

The dialysis was performed with synthetic biocompatible non-complement-activating polysulfone membranes (FX60 dialyzer; Fresenius Medical Care, Bad Homburg, Germany). The adequacy of dialysis was measured by the urea reduction ratio (>65%) and Kt/V (>1.2) values. DNA damage was evaluated using the MN frequency test in PBLs, three times before start of the study and three times during treatment with candesartan in 6-week intervals. The candesartan doses were prescribed according to the individual BP measurement with the intention to adjust it to upper normal levels. In dialysis patients, lower levels of BP are associated with a high risk of hypotension as a consequence of volume contraction during dialysis treatment. The BP was measured immediately before and during the hemodialysis sessions. The candesartan doses ranged between 4 and 16 mg/day with a low average level of 8.8 mg/day. The total treatment period lasted 4.5 months.

Blood Sampling

Venous blood samples were taken in the morning before start of dialysis, after a 2-day weekend break. Nine milliliters of whole blood was collected into heparinized containers (Sarstedt, Nümbrecht, Germany). The lymphocyte isolation started within 4 h. After the first centrifugation, the plasma was transferred to 1-ml tubes and stored at –80°C for further analyses.

Blood Chemical Analysis

Routine chemistry [such as electrolytes, creatinine (Cr), urea, total protein (TP), albumin (Alb), and C-reactive protein (CRP)] was analyzed employing Vitros 250 analyzer (J&J, Rochester, N.J., USA) before and after 3 and 4.5 months of treatment.

Lymphocyte Isolation and Cultivation

PBLs were isolated from whole blood via gradient centrifugation (Histopaque 1077; Sigma Diagnostics, St. Louis, Mo., USA), washed twice with RPMI 1640 medium (Sigma-Aldrich, Taufkirchen, Germany) and resuspended in RPMI 1640 medium supplemented with 15% FCS, 1% L-glutamine, 1% Na-pyruvate, 1% MEM non-essential amino acid solution and penicillin/streptomycin. The PBLs were cultured at 37°C in a humidified atmosphere containing 5% CO₂.

Micronucleus Frequency

MN are expressed in dividing cells that either contain chromosome breaks (resulting from unrepaired double-strand breaks) or by whole chromosomes that are unable to travel to the spindle poles during mitosis. They are observed in cells with completed nuclear division and are counted in the binucleated stage of the cell cycle after using the cytokinesis inhibitor cytochalasin B. This test was carried out as described by Fenech [35] with slight modifications. During the treatment period, cytochalasin B (5 µg/ml final concentration) was added to obtain binucleated cells. Human PBLs were cultured with phytohemagglutinin at a concentration of 5 µg/ml in order to stimulate the cells to enter the cell cycle. After 44 h, cytochalasin B was added. Following an additional 20 h of cultivation, cells were transferred to glass slides by cytospin centrifugation and fixed with methanol (-20°C, 1 h). For staining binucleated cells, the slides were incubated with acridine orange (62.5 µg/ml in Sorensen buffer, pH 6.8) for 5 min, washed twice with Sorensen buffer for 5 min and mounted for microscopy. The incidence of MN was obtained after scoring 1,000 binucleated cells per slide, with each data point representing the mean from 3 slides. In addition, the percentage of binucleated cells was evaluated as a cell proliferation marker. All counting of MN during the study was performed by the same person.

Statistics

Results are expressed as mean ± SEM. ANOVA with post-hoc Bonferroni was used to detect differences between groups. The Wilcoxon signed-rank test was used to analyze the development of parameters in matched groups. ANOVA with replication and post-hoc Bonferroni was used to evaluate differences in parameters observed over time. Statistical significance was defined starting at $p \leq 0.05$.

Results

Co-administration of candesartan resulted in a non-significant trend to lower BP values (table 2). There was no clear dose-dependent effect of candesartan; even a low dose of 4 mg/day decreased the systolic BP. In the conventionally treated MHD patients no change of BP during the time span of the study was observed (table 2).

Table 2. BP values and laboratory parameter before and after candesartan treatment

		Candesartan-treated MHD (n = 15)	Conventionally treated MHD (n = 14)
Systolic BP, mm Hg	before	148 ± 9	136 ± 24
	after	141 ± 9	132 ± 24
Diastolic BP, mm Hg	before	81 ± 11	71 ± 15
	after	78 ± 10	70 ± 12
Serum creatinine	before	11.1 ± 2.8	8.9 ± 2.0 [#]
	after	11.4 ± 2.4	8.9 ± 1.8 [#]
Serum urea, mg/dl	before	143 ± 20	120 ± 24
	after	137 ± 14	127 ± 22
Potassium level, mmol/l	before	5.1 ± 0.7	5.3 ± 0.7
	after	5.2 ± 0.4	5.3 ± 0.6
Albumin concentration, g/l	before	36 ± 3	39 ± 2
	after	37 ± 2	39 ± 3
Total protein, g/l	before	59 ± 5	60 ± 8
	after	59 ± 5	61 ± 6
CRP, mg/l	before	16.3 ± 10.6	13.0 ± 10.9
	after	16.9 ± 9.6	18.8 ± 16.0 [*]

* $p < 0.05$ vs. baseline.

[#] $p < 0.05$ vs. candesartan-treated MHD patients.

No changes in concentrations of serum creatinine, albumin or total protein were observed in either group throughout the treatment (table 2). The plasma concentration of potassium tended to increase in the candesartan-administered patients, while in those on conventional treatment it remained unchanged. In the treated group, the CRP levels did not change, while in the conventionally treated MHD patients, CRP rose significantly (table 2).

Micronuclei Frequency in Peripheral Blood

Lymphocytes

When compared to the healthy controls, the MNF was significantly higher before the start of the study in both MHD groups (fig. 1). Co-administration of candesartan ameliorated the degree of genomic damage by 27.7% ($p = 0.01$), while in the conventionally treated MHD controls the damage persisted.

The time course of the genomic damage in the candesartan-treated and conventionally treated groups is given in figure 2. Evidently, the beneficial effect of candesartan manifested itself already during the first 6 weeks of treatment and continued to persist over the whole study period.

Fig. 1. MNF/1,000 binucleated (BN) cells in healthy volunteers ($n = 14$) and in the candesartan (Cand)-treated ($n = 15$) and -untreated ($n = 14$) groups of MHD patients. * $p < 0.05$, ** $p < 0.01$ vs. healthy volunteers; ## $p < 0.01$ vs. Cand-treated MHD patients.

Fig. 2. Time course of MNF/1,000 binucleated (BN) cells in the MHD groups treated and untreated with candesartan (Cand). Values are shown after 6, 12 and 18 weeks. ** $p < 0.01$, *** $p < 0.001$ vs. micronuclei baseline of Cand-treated MHD patients.

Fig. 3. MNF/1,000 binucleated (BN) cells before and after 4.5 months of candesartan (Cand) therapy. Mean values of 3 measurement time-points before and after therapy in the individual patients ($n = 15$).

The individual data (mean values) of the MNF before and after candesartan treatment are presented in figure 3. In 11 of the 15 patients, a decline of MNF could be observed. The positive response was related to the baseline degree of MN, i.e. in the presence of highest micronuclei frequency levels the treatment effect was more pronounced. The lowering of MN was not related to the change of BP or the proinflammatory markers.

Discussion

Various therapeutic interventions improved the genomic damage in ESRD, e.g. co-administration of vitamin E [36], benfotiamine [37], folic acid and vitamin B₁₂ [38] resulted in a significant decline of micronuclei frequency. Moreover, daily dialysis was associated with less genomic damage [39].

In the current study, co-administration of candesartan for 4.5 months was reflected by a significant decline of the MNF, while in the conventionally treated MHD patients no change of DNA damage occurred. The beneficial effect was independent of BP changes. The decline of the BP was insignificant. This was due to the fact that the pretreatment values were only slightly elevated while the treatment target of BP values was in the upper normal range to prevent any dialysis-induced hypotension. The reduced micronuclei frequency in the candesartan-treated group suggests that an activated AT₁ receptor is involved in the development of genomic damage in ESRD. The fact that ANG II exerts genotoxic effects via the AT₁ receptor has been documented by our group in various mammalian cells [29], as well as in the perfused mouse kidney [40]. In addition, the marked accumulation of AGEs in ESRD could contribute to an activation of the AT₁ receptor, as demonstrated in our in vitro studies with various AGEs [27–29]. The lowering of DNA damage by candesartan is most probably a consequence of a decline of intracellular oxidative stress following a diminished activation of

NADPH oxidase. In our study no measurements of intracellular oxidative stress parameters were performed, but administration of enalapril to hemodialyzed patients resulted in the improvement of antioxidative defense [32]. In the present study the extracellular marker of inflammation, the acute phase reactant – CRP concentration – remained unchanged in the candesartan-treated group. However, in the untreated patients there was a significant rise of CRP. These results suggest an anti-inflammatory action of candesartan in our patients. A more pronounced anti-inflammatory action has been observed in patients with essential hypertension [41, 42], diabetics [43], or hypercholesterolemic volunteers [44]. However in these investigations the candesartan doses were much higher and up to 32 mg/day in contrast to our investigations with an average dose of 8.8 mg/day.

In the past it was shown that DNA damage plays a critical role in the mechanisms of atherosclerosis [11, 12] and cancer development [13, 14]. Long-term studies in larger patient groups are needed to evaluate whether the improved genomic damage observed in our study contributes to a decline in cardiovascular complications and cancer incidence. Interestingly, it was demonstrated that administration of the AT1 receptor blocker, candesartan, as well as of various ACE inhibitors in MHD patients resulted in a significant lowering of morbidity and mortality [33, 34]. However, a recent meta-analysis reported other findings in MHD patients: although treatment with ACE inhibitors or angiotensin receptor blockers reduced left ventricular mass, they were unable to reduce fatal and non-fatal cardiovascular events significantly [45].

It remains to be clarified whether the high incidence of cancer in MHD patients [18, 19] may be lowered by a long-term renin-angiotensin system blockade. In a cross-sectional study in hypertensive patients without renal

failure, long-term use of ACE inhibitors was shown to protect against cancer development [46]. However, in elderly hypertensive patients (mean age 76 years) a longitudinal, prospective study over a treatment period of about 5 years did not show any difference in cancer risk between patients assigned to conventional antihypertensive therapy or to ACE inhibitors [47]. To our knowledge, no data are available about a long-term effect of AT1 blockade on cancer risk.

Summarizing our data shows a BP-independent improvement of genomic damage in peripheral lymphocytes in MHD patients after co-administration of the AT1 receptor blocker, candesartan, for 4.5 months. If these alterations are representative of other tissues and the cardiovascular system, in the long run the lowered genomic damage could lead to beneficial effects of renin-angiotensin system blockade on morbidity and mortality in MHD patients.

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Disclosure Statement

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