Acute Effects of Haemodialysis on Pro-/Anti-Apoptotic Genes in Peripheral Blood Leukocytes

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Key Words

Apoptosis • Haemodialysis • Quantitative real-time PCR

Abstract

Several studies have implicated a remarkable dysfunctional apoptotic state and/or response in ESRD patients. Previously published studies are controversial with respect to acute effects of haemodialysis (HD) treatment on up- or downregulation of apoptotic genes. Twenty-eight chronic HD patients were haemodialysed for 4 hours with a 4008 dialyser using high-flux membranes. For subgroup analysis, patients were separated into a low (up to 0.5 mg/dl) and a high (0.5 to 5.0 mg/dl) CRP group. Blood was drawn prior to HD and 240 min after initiation of HD. Acute changes of transcript levels encoding pro- or anti-apoptotic genes were analyzed in RNA immediately isolated from blood leukocytes using quantitative real-time PCR. In the present study, we detected a significant elevation of the death receptor CD95/Fas (induction factor (IF) 1.55 ± 0.16), the death receptor 5 (DR5) (IF 1.17 ± 0.08), and caspase 8 (IF 1.37 ± 0.14) gene expression during HD. mRNA levels of the respective ligands

(CD95L, TRAIL), of the caspase 5 and anti-apoptotic Bcl-2 family members such as Bcl-2 and Bcl2l2 were slightly, but not significantly, increased after HD treatment. An additional anti-apoptotic molecule, BAG3, was found to be slightly, but significantly, induced after HD (IF 1.16 ± 0.07). In addition to being an activator of immune cells, CD40L has been shown to be strongly induced after HD treatment (IF 1.70 ± 0.20). Subgroup analysis revealed no significant differences between low vs. high CRP patient groups or diabetic vs. non-diabetic patients. These results indicate a marked influence of routine haemodialysis treatment on the transcription of pro- and anti-apoptotic molecules and the involvement of the extrinsic pathway for apoptosis through the activation of death receptors and the initiator caspase 8. Furthermore, following dialysis, lymphocytes seem to be activated by CD40L, which represents an early T-cell activation marker.

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Introduction

End-stage renal disease (ESRD) patients treated by standard haemodialysis are intermittently exposed to dialysis water and artificial surfaces. They are often in a

state of immunodeficiency, which may manifest as impaired polymorphonuclear leukocyte (PMN) function, but little is known about how haemodialysis may contribute to this process [1]. Apoptosis is a highly regulated process that mostly affects cell-mediated immunity, involving lymphocytes and monocytes, which play important roles in the immunologic defense of the host [2]. Several studies have suggested the existence of a remarkable dysfunctional apoptotic state and/or response in uremic patients [3-6]. PMN myeloperoxidase activity and apoptosis were increased in all renal failure patients in one study [7]. Sardenberg et al. investigated the effect of uremia and dialysis modality on apoptosis and function in freshly harvested non-incubated PMNs [8]. Their results suggest that both uremia and the treatment modality may interfere with PMN apoptosis and function [8].

However, controversial studies have been published on the acute effects of HD on the regulation of apoptotic markers or the impact of the used dialyzer membranes: Cell-free plasma DNA concentrations, annexin V expression, and 7-amino-actinomycin D uptake were described to be induced in leukocytes during haemodialysis [9]. Koller et al. demonstrated that apoptosis of human PMNs was accelerated by bioincompatible cuprophane membranes [10]. Soriano et al. found that in dialysis patients lymphocyte apoptosis is influenced not only by the biocompatibility, but also by the permeability of the dialysis membrane [11]. Other groups presented data that dialysis appears to normalize the increased PMN apoptosis rates observed in CKD patients [8]. In PMN cells, Andreoli et al. did not find an impact of dialyzer biocompatibility on PMN apoptosis [12]. The influence of two different dialysis membranes on apoptotic markers during HD was studied by flow cytometry [13]. In this study, HD patients had higher CRP, CD95 (Fas), and TNF-R2 (CD120b) baseline levels when compared with healthy controls. However, these levels remained the same during the HD sessions, and there were no significant differences between the two membranes [13]. Uremia and membrane characteristics may independently affect the mononuclear cell apoptosis [14].

The first event of the apoptotic cascade is the intracellular transcriptional mRNA up-regulation of pro-/anti-apoptotic genes in circulating leukocytes. We previously showed a marked influence of a routine haemodialysis treatment on transcription of pro- and anti-inflammatory cytokines and chemokine/cytokine receptors in leukocytes [15]. The aim of this study was thus to investigate responses in mRNA expression of

apoptotic genes during haemodialysis treatment.

Materials and Methods

Patients

Twenty-eight stable HD patients (16 male; 12 female; mean age 67 ± 10 years) from one dialysis centre were included in this study. Inclusion criteria were an adapted single-pool Kt/V urea >1.2 during HD session, normotensive predialytic systolic blood pressure (between 120 to 135 mmHg) and capable to understand information / consent form. Exclusion criteria were severe comorbidities (e.g., malignancy, active allergies), blood hemoglobin level <9 g/dl, C-reactive protein >5.0 mg/dl, patients do not comply with the protocol demands (e.g. dialyzer, blood samples) and active infections as indicated by fever $>38^{\circ}\text{C}$ or other symptoms. All patients gave informed consent, and the study was approved by the Ethics committee of the University of Tübingen.

For subgroup analysis, patients were divided into two groups: a low CRP group (CRP levels between 0.0 – 0.5 mg/dl, $n = 14$) and a high CRP group (CRP levels between 0.5 - 5.0 mg/dl, $n = 14$). Diabetic patients ($n = 13$) were also divided into a high CRP ($n = 7$) and a low CRP group ($n = 6$). Causes of renal insufficiency were diabetic nephropathy ($n = 13$), hypertensive nephropathy ($n = 4$), chronic glomerulonephritis ($n = 1$), vasculitis ($n = 2$), hereditary renal diseases ($n = 3$), and other causes ($n = 5$). For details of patient characteristics, see Table 1.

All patients were haemodialysed using a Fresenius 4008 dialysis machine (Fresenius Medical Care, Bad Homburg, Germany) containing a diasafe device for water pureness and using polysulfone high-flux dialyzer membranes (Fx60 / Fx80, Fresenius Medical Care, Bad Homburg, Germany). Bicarbonate dialysate was used throughout. Blood samples were drawn at 0 and 240 min. of the 4 hour HD sessions and after a two or three day dialysis free interval. Differential blood count, CRP, albumin, and total protein were measured using standard methods at our central laboratory unit.

PaxGene® RNA isolation and cDNA transcription

For the exact quantification of transcript levels, an RNA stabilizing system was required (for details [15]). Total RNA of blood leukocytes was immediately stabilized and extracted using the PaxGene System® (Qiagen, Hilden, Germany). Subsequently, 1 µg of total RNA was reverse transcribed into cDNA utilizing the reverse transcription system (Bioscience, USA) with oligo(dT) primers according to the manufacturer's protocol.

Quantitative real-time PCR

To quantify mRNA expression, quantitative real-time PCR with the LightCycler System (Roche Diagnostics, Mannheim, Germany) was used (for details [16]). Real-time PCR analyses of all genes were performed in a final volume of 20 µl containing 2 µl cDNA, 2 µl primer mix (Search LC, Heidelberg, Germany), 2 µl DNA Master Sybr Green I mix (Roche Molecular

	All patients (n = 28)	Low CRP- Group (n = 14)	High CRP Group (n = 14)	Statistical analysis (low / high)
Age (years)	67 ± 10	65 ± 11	68 ± 9	n.s.
Time on haemodialysis (months)	52 ± 57	64 ± 75	41 ± 34	n.s.
Dialyzer membranes (Fx60 / Fx80)	26 / 2	13 / 1	13 / 1	n.s.
Diabetes mellitus (type 2)	13 / 28	6 / 14	7 / 14	n.s.
Peripheral arterial occlusive disease	12 / 28	4 / 14	8 / 14	< 0.05
Haemoglobin (g/dl)	11.1 ± 1.0	11.3 ± 1.0	10.9 ± 1.1	n.s.
Leukocytes / μ l	6408 ± 2088	5691 ± 1622	7125 ± 2306	n.s.
CRP (mg/dl)	0.9 ± 1.1	0.2 ± 0.14	1.7 ± 1.2	< 0.01
Albumin (g/dl)	3.9 ± 0.4	4.1 ± 0.3	3.7 ± 0.4	n.s.
Total protein (g/dl)	6.9 ± 0.5	6.9 ± 0.5	6.8 ± 0.6	n.s.

Table 1. Patient characteristics

Biochemicals, Mannheim, Germany), and 14 μ l DEPC treated water. Amplification of the target DNA was performed for 35-38 cycles of 95°C for 10s, 68°C for 10s, and 72°C for 16s, each with a temperature transition rate of 20°C/s and a secondary target temperature of 58°C with a step size of 0.5°C. Melting curve analysis was performed at 95°C 0s, 58°C 10s, and 95°C 0s to allow discrimination between nonspecific primer dimers and specific PCR products. The transcript levels of the housekeeping gene GAPDH as well as CD95 (Fas), CD95L (FasL), DR5 (TRAILR2), TRAIL, Caspase 5, Caspase 8, CD40, CD40L, Bcl-2, Bcl-2L2, and BAG3 were determined for each sample using commercial primer kits (Search LC, Heidelberg, Germany). Melting curve analysis confirmed the amplified products, which were then separated on 1.5% agarose gels to confirm the expected size. Induction factors were calculated as a ratio between the target genes and house keeping gene GAPDH compared with transcript levels before HD.

Statistical Analysis

Data are provided as means $\pm$ SEM, n represents the number of patients, all data were tested for significance using ANOVA or t-tests, as appropriate, and only results with $p < 0.05$ were considered statistically significant.

Results

Haemodialysis patients

To examine the influence of the microinflammatory status on apoptotic genes, haemodialysis patients with high CRP levels (average 1.7 ± 1.2 mg/dl) and patients with low CRP levels (average 0.2 ± 0.1 mg/dl) were analyzed separately. In both CRP groups, patients with diabetes mellitus type 2 (high: 7 out of 14 vs. low: 6 out of 14, n.s.) were included. The high CRP group had significantly more patients with peripheral arterial occlusive disease compared to the low CRP group (8 out of 14 vs. 4 out of 14, $p < 0.05$). Blood leukocyte count was not significantly

Fig. 1. CD95 (Fas)/CD95L (FasL) transcript levels. Induction factors were calculated as ratio between target genes and house keeping gene GAPDH compared with transcript levels before HD from all patients (n = 28), the high CRP group (n = 14), and the low CRP group (n = 14). The diabetic patients (n = 13) were also divided into a high CRP (n = 7) and low CRP group (n = 6). Values are shown as mean $\pm$ SEM. (A): CD95 (B): CD95L. * Significant induction versus transcript levels before HD ($p < 0.05$).

Fig. 2. Caspase 5/Caspase 8 transcript levels. Induction factors were calculated as a ratio between target genes and house keeping gene GAPDH compared with transcript levels before HD from all patients (n = 28), the high CRP group (n = 14), and the low CRP group (n = 14). The diabetic patients (n = 13) were also divided into a high CRP (n = 7) and low CRP group (n = 6). Values are shown as mean $\pm$ SEM. (A): Caspase 5 (B): Caspase 8. * Significant induction versus transcript levels before HD ($p < 0.05$).

Fig. 3. CD40/CD40L transcript levels. Induction factors were calculated as a ratio between target genes and house keeping gene GAPDH compared with transcript levels before HD from all patients (n = 28), the high CRP group (n = 14), and the low CRP group (n = 14). The diabetic patients (n = 13) were also divided into a high CRP (n = 7) and low CRP group (n = 6). Values are shown as mean $\pm$ SEM. (A): CD40 (B): CD40L. * Significant induction versus transcript levels before HD ($p < 0.05$).

higher in the high CRP group ($7125 \pm 2306/\mu\text{l}$) than in the low CRP group ($5691 \pm 1622/\mu\text{l}$). HD patients in the two groups did not differ significantly from each other with respect to age, time on haemodialysis, type of dialyzer membranes used, or plasma concentrations of haemoglobin, albumin, or total protein. Clinical conditions of the groups are provided in Table 1.

Quantitative real-time PCR

Transcript levels of CD95 (Fas) / CD95L (FasL)

Including all patients, the induction factor (IF) of CD95 (Fas) transcript levels to GAPDH mRNA levels

significantly increased by 1.55 ± 0.16 following a 240 min HD treatment ($p < 0.05$, Fig. 1a). When patients were separated into a high and a low CRP group, CD95 levels were significantly higher in the low CRP group following HD (1.81 ± 0.25 fold, $p < 0.05$), in comparison to levels obtained prior to HD. However, transcript levels were not significantly different between the low/high CRP subgroups. Comparable induction factors were found in diabetic patients (Fig. 1a).

HD treatment was also followed by a slight increase in transcript levels of CD95L (FasL) in the pooled patient group (1.34 ± 0.19), which was not significant (Fig. 1b).

Fig. 4. DR5/BAG3 transcript levels. Induction factors were calculated as a ratio between target genes and house keeping gene GAPDH compared with transcript levels before HD from all patients ($n = 28$), the high CRP group ($n = 14$), and the low CRP group ($n = 14$). The diabetic patients ($n = 13$) were also divided into a high CRP ($n = 7$) and low CRP group ($n = 6$). Values are shown as mean $\pm$ SEM. (A): DR5/TRAILR2 (B): BAG3. * Significant induction versus transcript levels before HD ($p < 0.05$).

Fig. 5. Bcl-2/Bcl-2L2 transcript levels. Induction factors were calculated as a ratio between target genes and house keeping gene GAPDH compared with transcript levels before HD from all patients ($n = 28$), the high CRP group ($n = 14$), and the low CRP group ($n = 14$). The diabetic patients ($n = 13$) were also divided into a high CRP ($n = 7$) and low CRP group ($n = 6$). Values are shown as mean $\pm$ SEM. (A): Bcl-2 (B): Bcl-2L2. No significant induction was measured versus transcript levels before HD.

The observed differences within the subgroups (high CRP vs. low CRP) were not significant due to high interindividual variations.

Transcript levels of Caspase 5 and Caspase 8

HD treatment led to an increase of caspase 5 (1.35 ± 0.19 ; n.s. - Fig. 2a) and caspase 8 (1.37 ± 0.14 , $p < 0.05$) transcript levels in the pooled patient group (Fig. 2b). An increase of transcript levels encoding caspase 5 was detected only in the low CRP group (1.62 ± 0.36 , n.s. - Fig. 2a). Caspase 8 was slightly increased in the high CRP group (1.20 ± 0.14 , n.s.) and in the low CRP group (1.53 ± 0.25 , n.s. - Fig. 2b). No significant differences could be measured between

CRP subgroups or diabetic/non-diabetic patients.

Transcript levels of CD40 and CD40L

No significant alteration of CD40 transcript levels could be found in the pooled patient group or the subgroups after HD treatment (Fig. 3a). In contrast to these results, CD40L was significantly enhanced in the pooled group of patients (1.70 ± 0.20 , $p < 0.05$ - Fig. 3b) and in the low CRP group (1.77 ± 0.17 , $p < 0.05$). The increase in CD40L transcript levels was more pronounced in the non-diabetic group than in the diabetic patient group, but the difference did not reach statistical significance. Furthermore, there was no significant difference between the high/low CRP groups.

Transcript levels of DR5/TRAILR2 and TRAIL

Transcript levels of DR5/TRAILR2 were slightly, but significantly, increased (1.17 ± 0.08) in the pooled patient group ($p < 0.05$, Fig. 4a). The induction was comparable in the diabetic and in the high/low CRP subgroups, and there were no statistically significant differences among these groups. Transcript levels of TRAIL did not differ significantly after HD treatment (data not shown).

Transcript levels of Bcl-2, Bcl-2L2, and BAG3

Bcl-2 was slightly increased in the pooled patient group (1.24 ± 0.15 , n.s.), in the high CRP group (1.26 ± 0.19 , n.s.), and in the low CRP group (1.22 ± 0.23 , n.s. - Fig. 5a) without reaching statistical significance. No significant differences were observed between CRP subgroups or diabetic/non-diabetic patients. No alteration of Bcl-2L2 transcript levels could be found after HD treatment (Fig. 5b). In contrast to these results, HD treatment led to a significant increase in BAG3 transcript levels in the pooled patient group (1.16 ± 0.07 ; $p < 0.05$) and the high CRP group (1.27 ± 0.11 , $p < 0.05$), but not in the low CRP group (1.07 ± 0.09 ; n.s. - Fig. 4b). No significant differences were observed between CRP subgroups or diabetic/non-diabetic patients.

Discussion

Several studies have implicated a remarkable dysfunctional apoptotic state and/or response in end-stage renal disease patients [17, 18]. In vitro data have shown that neutrophils from uremic patients undergo accelerated apoptosis [19] and uremic plasma obtained from HD patients accelerates apoptosis in PMNs from healthy donors [20]. In haemodialysis (HD) patients, it is still unclear whether increased apoptosis is due to uraemia or HD treatment itself [21]. Inconsistent results have been published with respect to *acute* effects of HD treatment on up- or downregulation of apoptotic genes [8, 9, 11-13].

In the present report, we carried out a quantitative analysis of the apoptotic gene expression profile in blood leukocytes before and after a standard haemodialysis treatment using a polysulfone high-flux membrane. For reliable quantification of transcript levels in blood leukocytes, a RNA stabilizing system was established (for details [15]). We detected a significant elevation of three components of the extrinsic pathway for apoptosis after a standard 4-h haemodialysis treatment: the death

receptors CD95/Fas, DR5, and caspase 8, indicating that the extrinsic pathway is activated during HD treatment and may trigger apoptosis. mRNA levels of CD95L (FasL) were slightly increased after HD treatment; however, no significant differences were observed. Subgroup analysis revealed no significant difference between diabetic vs. non-diabetic patients or high- vs. low-CRP levels in any of the examined genes. Known as the death receptor pathway, the extrinsic or caspase 8/10-dependent pathway is activated by ligand binding of pro-apoptotic molecules [22]. The death receptors are specialized cell-surface receptors including CD95 (Fas), tumor necrosis factor- α receptor 1 (TNF- α R1), and two death receptors, DR4 and DR5, that bind to the TNF- α related apoptosis-inducing ligand (TRAIL) [23, 24]. In our previous work, we demonstrated that TNF- α is significantly upregulated in leukocytes during a standard 4-h haemodialysis session [15]. TNF- α binds to the TNF receptors and activates the extrinsic pathway for apoptosis via the intermediate proteins TNF receptor-associated death domains (TRADD) and Fas-associated death domains (FADD). This leads to caspase 8 activation [22]. It is well known that CD95 (Fas) expression is significantly higher in uremic patients compared with control subjects, HD patients, or PD patients [25, 26]. Our results are in line with data that demonstrated that haemodialysis treatment itself promotes apoptosis via CD95 (Fas) [27]. On the other hand, recent data did not show significant differences in CD95 (Fas) protein levels during haemodialysis on T and B lymphocytes or on monocytes [13]. Changes of protein levels during HD are probably difficult to capture at early time points; for this reason, we decided to measure the intracellular transcriptional regulation of apoptotic genes in leukocytes. It should be kept in mind, though, that transcription of cytokines does not necessarily reflect the respective cellular protein release.

A slight, but significant increase of mRNA copy numbers for death receptor 5 (DR5/TRAILR2) was observed after haemodialysis treatment. The protein encoded by DR5 is a member of the TNF-receptor superfamily and contains an intracellular death domain. This receptor can be activated by TNF-related apoptosis inducing ligand (TNFSF10/TRAIL) and transduces the apoptosis signal. The current study represents the first report that DR5 transcription is enhanced after HD treatment. Transcript levels of TRAIL did not differ significantly after HD treatment.

CD40L is a member of the tumor necrosis factor (TNF) family of cell surface interaction molecules [28]. The expression is induced shortly after T-cell activation

and represents an early activation marker of T lymphocytes. T cell receptor (TCR) ligation initiates the induction of CD40L expression on the surface of the activated T cells. Additional costimulatory signals (IL-2, IL-12, IL-15) enhance CD40L upregulation, while others, such as IL-4 and IL-10, downregulate CD40L [28]. Previously, we detected significantly higher transcription levels of the IL-4 and IL-10 receptors in low CRP patients after HD treatment [15]. This seems surprisingly, while CD40L was significantly enhanced in the pooled group of HD patients and in the low CRP subgroup.

After T-cell stimulation, CD40L mRNA expression peaks at 1 to 2 h, and cell surface CD40L protein is fully expressed within 4 to 6 h [29]. This indicates that that expression profile analyses on mRNA levels during HD are more reasonable than those on protein levels. CD40L gene expression has been demonstrated to increase fourfold in cases of acute renal allograft rejection [30]. Based on this, the detected 1.7-fold increase of CD40L in our study during one HD session appears to be relatively high. Furthermore, slight induction levels are not surprising because gene expression levels of all leucocytes were analysed without previous separation of lymphocyte subsets.

Activation of CD40 is associated with a broad range of biological effects depending on the cell type and nature of the stimulus. For example, CD40 on antigen-presenting cells provides a vital signal for T cell activation and proliferation, whereas activation of CD40 on the endothelium results in a proinflammatory signal that leads to chemokine secretion and increased adhesion molecule expression [31]. Activation of the CD40 receptor by its cognate ligand, CD40L or CD154, results in interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1) production and increased intercellular adhesion

molecule-1 (ICAM-1) expression in proximal tubule cells (PTCs) [32]. In our previous study, we detected strongly enhanced IL-8 transcript levels in blood leukocytes after HD treatment, which could be a result of the CD40L activation found in the present study [15]. Interestingly, in addition to being an activator of immune cells, CD40L has been shown to induce apoptosis in tumor cells [33].

Bcl-2 is the prototype for a family of mammalian genes that govern mitochondrial outer membrane permeabilization (MOMP) and can be either pro-apoptotic (Bax, BAD, Bak, and Bok among others) or anti-apoptotic (including Bcl-2, Bcl-xL, and Bcl-w, among an assortment of others) [34]. The proteins of the Bcl-2-like 2 family form hetero- or homodimers and act as anti- and pro-apoptotic regulators [34]. Expression of this gene in cells has been shown to contribute to reduced cell apoptosis under cytotoxic conditions. Reports regarding the effect of HD on Bcl-2 are controversial [35]. Gene expression levels of the analyzed Bcl-2 and Bcl-2L2 in our study achieved no significant increase during the HD treatment. However, BAG3 levels showed a slight, but significant, elevation during this time. The BAG domains of BAG1, BAG2, and BAG3 interact specifically with the Hsc70 ATPase domain in vitro and in mammalian cells [36]. However, it remains unclear whether their effects are sufficient to counteract the pro-apoptotic signals during HD.

Taken together, the results of the present work indicate an upregulation of pro- and anti-apoptotic molecules during HD treatment. The involvement of the extrinsic pathway through significant elevation of two death receptors (CD95 and DR5) and of caspase 8 plays a central role in the initiation of the execution-phase of cell apoptosis. CD40L induction during HD may result in a pro-inflammatory T-cell activation.

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