Production of monokines in patients under polysulphone haemodialfiltration is influenced by the ultrafiltration flow rate

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Abstract
Background. Chronic haemodialysis patients show various clinical signs of immunodeficiency and there is growing evidence that a dysregulated monocyte cytokine production is heavily involved in this deficiency. The production of monokines in vitro has been proposed to correlate closely with the in vivo immune status and to be of high clinical relevance in cuprophone haemodialysis. Even though it is well known that the biocompatibility of dialyser membranes has a significant impact on immune functions, little is known about the influence of the ultrafiltration flow rate (UFR). The aim of this study was to investigate the potential long-term effects of UFR on the production of interleukin-10 (IL-10), interleukin-1β (IL-1β) and interleukin-6 (IL-6) in an intra-individual study design.

Methods. In 11 patients previously treated with polysulphone haemodialfiltration, UFR was reduced from 40–46 ml/min to 24–28 ml/min, then to 7–10 ml/min before it was reinstated at 40–46 ml/min for periods of 4 weeks each. Monokine secretion into culture supernatants and mRNA expression (assessed using a novel Taqman PCR technique), were determined in a whole blood assay after lipopolysaccharide stimulation.

Results. Reduction of UFR led to a significant increase in IL-10 secretion and mRNA expression (P = 0.012, P = 0.001). Conversely, a substantial (but not complete) decrease was observed when UFR returned to initial levels. In contrast, supernatant concentrations of IL-1β (P = 0.04) and IL-6 (P = 0.003), and mRNA expression of both monokines (P < 0.001, P < 0.001) decreased significantly when UFR was reduced. Calculation of the IL-1β/IL-10 ratio also revealed a decrease when UFR was reduced, with an increase again being observed when the initial degree of UFR was reinstated (P < 0.001).

Conclusions. These results indicate a significant impact of UFR on the production of monokines at both the transcriptional and the protein level. We suggest that middle molecule removal has to be considered as a possible pathophysiological mechanism to explain our findings. Since monokine production in vitro was shown to be closely correlated with the in vivo immune status in patients on cuprophone haemodialysis, further investigations are necessary to clarify the impact of UFR on the immunocompetence of patients under polysulphone haemodialfiltration.

Keywords: haemodiafiltration; immunocompetence; monokines; ultrafiltration flow rate

Introduction

Haemodialysis patients show clinical signs of immunodeficiency, such as non-responsiveness to vaccinations and higher mortality from infections [1,2]. Besides uraemia, haemodialysis treatment itself has a significant impact on immune functions, and the biocompatibility of dialyser membranes in particular was found to be a crucial influencing parameter [3,4]. In addition, several authors indicated an independent impact of middle-molecule removal on the risk of mortality in haemodialysis patients [5,6] and inhibitory effects of middle molecules on immune cells could be demonstrated [7]. Since the elimination of middle molecules is directly correlated to ultrafiltration flow rate (UFR) [8], it was the central aim of the current study to investigate a possible impact of UFR on key immune functions.

An impaired activation of T-lymphocytes by accessory cells such as monocytes seems to play a key role in dialysis related immunodeficiency [9]. As alterations
in monokine production represent a common finding in haemodialysis patients, it was recently found that the production of regulatory (IL-10) and proinflammatory monokines (IL-1β, IL-6) in vitro correlates closely with the immune defect and is therefore of high clinical relevance [10,11].

The current study examines the impact of UFR on the monokine response in an intra-individual study design. As the separation of monocytes from a physiological environment is likely to have profound modifying effects on cell function and can cause pre-activation [12], the induction of IL-10, IL-1β and IL-6 upon lipopolysaccharide (LPS) stimulation was investigated under whole blood conditions in vitro. Monokine secretion into culture supernatants was compared to mRNA expression measured using a novel Taqman PCR-technique [13].

Subjects and methods

Patients and study design

Seven male and four female patients (age 23–78 years, median 55 years) with end-stage renal disease on regular haemodialfiltration treatment were studied. Chronic renal failure was due to glomerulonephritis in three cases, end-stage renal disease of uncertain aetiology in three cases, polycystic kidney disease in two cases, diabetes mellitus in one case, Alport syndrome in one case, and analgesic nephropathy in one case. Patients with chronic inflammatory or systemic diseases were excluded. Active infections or inflammatory states were ruled out by determining body temperatures, C-reactive protein levels (threshold 5.0 mg/l), and blood counts. Participating patients were not taking any immunosuppressive medications and showed no signs of obvious malignancy. Patients were treated with recombinant human erythropoietin at an average dose of 95 IE/kg body weight/week when the haematocrit was below 32%. The median duration of haemodialysis prior to the study was 6 years (range 1–18 years). Patients underwent haemodiafiltration three times a week for 4–5 h and were dialysed with the same polysulphone high-flux membrane BLS 627c (Sorin Biomedica-Belco, Mirandola, Italy). Only new membranes were used and there was no reuse of the dialysers.

We selected patients with a blood flow of 250 ml/min and a minimum withdrawal of 2000 ml, to exclude the possibility of any relevant back-filtration. The dialysate flow was 500 ml/min. The UFR was calculated for each patient considering replacement fluid, withdrawal and dialysis time.

All participants had been haemodialysed at 9000 ml replacement fluid (UFR of 40–46 ml/min) for a minimum of 2 months before the onset of the study. Subsequently the replacement fluid was reduced to 4500 ml (UFR 24–28 ml/min), 0 ml (UFR 7–10 ml/min) and returned to 9000 ml (UFR 40–46 ml/min) at 4-week intervals. The efficiency of haemodiafiltration treatment and UFR reduction was verified by determination of urea and β2-microglobulin reduction ratio and β2-microglobulin serum concentrations. Only sterile and pyrogen-free replacement fluid was used containing 34.0 mmol/l lactate (Biosol, Sondalo, Italy). Analysis of the dialysate revealed sterile water (culture test) and endotoxin levels of <0.24 IU/ml (LAL test) throughout the surveillance period. Whole-blood samples were always collected immediately before haemodialysis treatment at the onset of the study and at the end of each of the 4-week periods. The study was approved by the Ethics Commission of the Lübeck University School of Medicine. Written informed consent was provided by all participants.

Whole-blood assay

To approximate the in vivo immune status, the production of monokines was determined in non-separated blood [14]. In brief, 200 ml lithium heparinized blood was mixed with 1600 ml culture medium (RPMI-1640 medium, Biochrom, Berlin, Germany) supplemented with 1% l-glutamine and 1% penicillin/streptomycin. 200 ml of LPS solution was added, so that a final concentration of 1 mg/ml LPS was achieved for inducing IL-10, IL-1β and IL-6. LPS-free cultures served as negative controls and were treated identically. Cultures were incubated under 5% CO2 in a humidified incubator at 37°C for 24 h.

Quantification of IL-10, IL-1β, and IL-6 in culture supernatants

Supernatants from whole-blood assays were frozen at −80°C prior to monokine determination. Values of IL-10, IL-1β and IL-6 were quantified by an enzyme immunoassay (EIA) technique (R&D Systems, Minneapolis, MN, USA). Determinations on all samples, negative controls and standards were performed in duplicate. According to information provided by the manufacturer, no cross-reactivity to any related cytokines was known.

Quantification of IL-10, IL-1β, and IL-6 mRNA copies by Taqman PCR

For quantifying monokine mRNA, a novel Taqman technique was applied that was recently established in our laboratory [13]. In brief, total RNA was isolated from the whole-blood cultures immediately after a 24-h incubation using the Purescript RNA isolation kit (Gentra Systems, Minneapolis, USA). Sequence-specific PCR primers and fluorochrome-labelled internal oligonucleotide probes for IL-10, IL-1β and IL-6 were designed using the ‘Primer express’ software (Perkin Elmer Cetus, Foster City, CA, USA). In order to use cDNA in the quantitative PCR, 30 μl of total RNA were reverse transcribed using a commercial kit (Ready to Go, Pharmacia, Uppsala, Sweden).

The PCR reaction mixture contained 5 μl 10× Taqman A-buffer (500 mmol/l KCl, 100 mmol/l Tris·HCl, 100 mmol/l EDTA, 600 mmol/l passive reference dye ROX at pH 8.3; Perkin Elmer, Foster City, CA, USA), 3.5 mmol/l MgCl2, 300 μmol/l dATP, dCTP, dGTP, 600 μmol/l dUTP, 100 nmol/l of forward and reverse primer, 100 nmol/l fluorogenic probe, U Ampli-Taq Gold-DNA-Polymerase (Perkin Elmer, Foster City, CA, USA), and 10 μl of water control, diluted standards or sample in a total volume of 50 μl. The PCR conditions were 10 min at 95°C for DNA-polymerase activation, followed by 40 cycles of 15 s at 95°C and 1 min 30 s at 60°C with a final 25°C hold.

In order to create standards, cDNA fragments were cloned that encoded β-actin, IL-10, IL-1β and IL-6. PCR-products were ligated into vector pCRH (Original TA Cloning Kit, Invitrogen, San Diego, CA), and used to transform
INVaF™ competent *E. coli* cells. The plasmid concentration was determined by measuring the optical density at wavelength 260 nm using a U-3000 spectrophotometer (Hitachi, Tokyo, Japan). All PCRs for monokine mRNA quantification were performed in an ABI PRISM 7700 Sequence Detector System (Applied Biosystems, Foster City, CA, USA). Standardized monokine mRNA quantities (monokine mRNA copies/10^6 β-actin copies) were determined by dividing the interpolated values derived from the monokine standard curve by the β-actin mRNA content (which served as a normalization factor). Analysis was performed in triplicate.

**Statistical analysis**

Statistical analysis was performed using commercially available software for personal computers (SSPS for Windows 8.0, SPSS GmbH, Munich, Germany). Concentrations of monokines in supernatants and numbers of mRNA copies/10^6 β-actin copies were reported as mean ± SD. The Friedman test and the appropriate post-hoc analysis (Wilcoxon Wilcox test) were used to compare non-parametrically distributed data. *P* values < 0.05 were considered significant.

**Results**

**Urea and β2-microglobulin reduction ratio and β2-microglobulin serum levels**

Determination of the urea reduction ratio revealed constant values throughout the study period, whereas the β2-microglobulin reduction ratio showed a direct correlation to the UFR. Compared with the onset of the study, it was significantly elevated, when 40–46 ml/min was reinstated (Table 1). In contrast, β2-microglobulin serum values increased with reduction of the UFR and decreased again when 40–46 ml/min was reinstated (*P* < 0.001) (Table 1).

**Determination of peripheral blood counts and C-reactive protein**

Analysis of peripheral blood counts revealed constant monocyte counts throughout the study period (*P* = 0.19), so that a possible influence on the observed results could be excluded: (UFR 40–46 ml/min, 0.473 ± 0.129 × 10^9/l; UFR 24–28 ml/min, 0.541 ± 0.212 × 10^9/l; UFR 7–10 ml/min, 0.486 ± 0.185 × 10^9/l, and UFR 40–46 ml/min, 0.444 ± 0.151 × 10^9/l). Likewise, C-reactive protein levels remained unchanged (*P* = 0.62): (UFR 40–46 ml/min, 1.0 ± 0.9 mg/l; UFR 24–28 ml/min, 1.0 ± 0.8 mg/l; UFR 7–10 ml/min, 0.9 ± 0.7 mg/l; and UFR 40–46 ml/min, 0.9 ± 0.8 mg/l).

**Secretion of IL-10, IL-1β, and IL-6 in whole-blood culture supernatants**

At the start of the study, all patients had been haemodialysed at a UFR of 40–46 ml/min for a minimum of 2 months. Before the UFR was reduced to 24–28 ml/min, 7–10 ml/min and returned to 40–46 ml/min, secretion of monokines was determined to provide initial values (Figure 1). While the stepwise reduction in UFR to 7–10 ml/min resulted in a significant increase in IL-10 concentrations (*P* = 0.012), the return to 40–46 ml/min resulted in a substantial, although incomplete, reversal of this effect. In contrast, IL-1β concentrations decreased in a stepwise manner (*P* = 0.04) before they increased only slightly after UFR was reinstated at 40–46 ml/min. Reduction of UFR also resulted in a lowering of IL-6 concentrations (*P* = 0.003), but there was no increase in IL-6 levels, when the high UFR was reinstated. Analysis of LPS-free whole-blood cultures and serum samples of the patients revealed that there was no spontaneous monokine secretion throughout the study.

**Quantification of IL-10, IL-1β, and IL-6 mRNA in whole-blood cultures**

The content of monokine mRNA was quantified by Taqman PCR. Copy numbers of monokine mRNA were calculated per 10^6 β-actin copies, whereby the latter served as a normalization factor to compensate for cell number variability. With reduction of the UFR, IL-10 mRNA copy numbers increased in a stepwise manner, and decreased again when the UFR was returned to 40–46 ml/min (*P* = 0.001) (Figure 2). In contrast, IL-1β and IL-6 mRNA decreased (*P* < 0.001, *P* < 0.001) with reduction of the UFR. When the original UFR was reinstated, IL-1β mRNA slightly increased again but there was no reversal of this effect for IL-6 mRNA.

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<tr>
<th>Table 1. Urea and β2-microglobulin reduction ratio and β2-microglobulin serum levels at different UFRs</th>
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<td><strong>Urea reduction ratio (%)</strong></td>
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<tr>
<td>UFR 40–46 ml/min</td>
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<tr>
<td>63.6 ± 7.8</td>
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<td>58.3 ± 12.0</td>
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While urea reduction remained constant (*P* = 0.97), β2-microglobulin reduction decreased in parallel with UFR and increased again when 40–46 ml/min was reinstated (*P* = 0.001). With reduction of the UFR, β2-microglobulin serum levels increased inversely and decreased again when 40–46 ml/min was reinstated (*P* < 0.001). All values are expressed as mean ± SD.

*Significant differences compared to initial values.
Fig. 1. Comparison of the effects of different UFRs on monokine concentrations in whole blood culture supernatants after 24 h of stimulation by LPS. Concentrations of IL-10 increased with reduction of the UFR to 24–28 ml/min and 7–10 ml/min respectively but then decreased when 40–46 ml/min was reinstated (P = 0.012). In contrast, IL-1β and IL-6 decreased with reduction of the UFR (P = 0.04, P = 0.003). *Significant differences compared with initial values.

In addition, the IL-1β/IL-10 ratio was calculated for the protein and the mRNA content (Figure 3). Both ratios declined in parallel with reduction of the UFR and increased when 40–46 ml/min was reinstated (P < 0.001, P < 0.001).

Discussion

There is growing evidence that a dysregulated monocyte cytokine production plays a crucial role in immune deficiency amongst haemodialysis patients.
Fig. 2. Comparison of the effects of different UFRs on monokine mRNA copies/10^6 β-actin copies after 24 h of stimulation by LPS. Concentrations of IL-10 mRNA increased with reduction of the UFR to 24–28 ml/min and 7–10 ml/min, but decreased again when a UFR of 40–46 ml/min was reinstated (P = 0.001). Copy numbers of IL-1β mRNA decreased with reduction in UFR (P < 0.001), but increased slightly when 40–46 ml/min was reinstated. IL-6 mRNA copy numbers decreased in parallel when the UFR was reduced (P < 0.001), but did not increase again when the higher UFR was reinstated. *Significant differences compared with initial values.

[15–17]. Although it is well known that the biocompatibility of dialyser membranes is a major factor that influences immunocompetence [3,4], little is known about the impact of the UFR on cytokine production in monocytes.

Our results indicate that UFR has significant long-term effects on the production of monokines at both the transcriptional and the protein level within the same individual. Thus an independent influence on the production of the regulatory monokine IL-10 upon
LPS stimulation but not spontaneous IL-10 secretion can be postulated. Subsequent reduction of UFR led to increasing concentrations of IL-10 in culture supernatants, whereas reinstatement of UFR at 40–46 ml/min resulted in a subsequent decrease in the values. This result was confirmed by a parallel increase and subsequent decrease in IL-10 mRNA copy numbers. Moreover, it proves that UFR influences the IL-10 synthesis at the transcriptional level. Since monocyte counts remained unchanged throughout the study and copy numbers of mRNA were divided by $10^6$ β-actin copies for normalizing the data, it can be concluded that the increased IL-10 production is due to an increased synthesis rather than an increase in monocyte counts.

This finding could be of great relevance, since IL-10 exerts important regulatory effects on monocytes. It strongly reduces antigen-specific T-lymphocyte proliferation by diminishing the antigen-presenting capacity of monocytes via downregulation of MHC class II expression and inhibits the production of proinflammatory monokines, such as IL-1β and IL-6 [18–20]. Accordingly, with reduction of the UFR, we found a stepwise reduction of IL-1β and IL-6 in our patients at both the protein and the mRNA level. Thus we suggest a dose-dependent relationship between the UFR and the production of IL-10 leading to a counterregulation of the pro-inflammatory monokines IL-1β and IL-6. Moreover, since the production of proinflammatory monokines upon LPS-stimulation in vitro was shown to correlate closely with clinical parameters [11], we postulate a direct impact of the UFR on the clinical immune status.

Comparison with healthy controls that were previously investigated in our laboratory under the same conditions [21] (data not shown), revealed identical levels of IL-6 in our patients prior to UFR reduction. Thus our data would be in accordance with recent findings that described comparable levels of proinflammatory monokines in patients treated with polysulphone haemodialysis and healthy controls [22].

The elimination of middle molecules must be regarded as a potential pathophysiological mechanism to explain our results, since immunomodulating effects of middle molecules have already been postulated [6]. In a recent study, a direct inhibition of lymphocyte
proliferation and IL-2 production was demonstrated [7], and it remains unclear whether this inhibition is restricted to lymphocytes or whether middle molecules affect immune functions in a rather unspecific fashion. Determination of $\beta_2$-microglobulin reduction ratio confirmed a direct correlation between the UFR and middle-molecule elimination in our patients [23], and reduction of UFR resulted in an increase of $\beta_2$-microglobulin serum levels and vice versa (Table 1). In contrast, the urea reduction ratio remained unchanged throughout the study. Therefore it should be clarified whether an accumulation of middle molecules in patients under polysulphone haemodialfiltration enhances the production of IL-10 leading to an inhibition of IL-1$\beta$ and IL-6. Interestingly, when a UFR of 40–46 ml/min was reinstated, the $\beta_2$-microglobulin reduction ratio and $\beta_2$-microglobulin serum levels were still elevated compared with the onset of the study. This could indicate either an increased $\beta_2$-microglobulin production, or that steady-state conditions were not achieved again within the observation period. Furthermore, it could explain why IL-1$\beta$ and IL-6 levels were still reduced. Since C-reactive protein levels remained constant and spontaneous monokine secretion into unstimulated controls was not detectable throughout the study, a direct influence of any undiscovered cytokine-inducing substances in the replacement fluid seems rather unlikely. Moreover, our findings indicate that the UFR could have an independent impact on key immune functions. This could be highly relevant, since the lower mortality in patients treated with haemodialfiltration has not been fully understood and the relative importance of middle-molecule removal vs membrane biocompatibility currently remains a matter of discussion [24,25]. Thus, additional investigations are required to clarify the impact of UFR on the immunocompetence of patients under haemodialfiltration.

In conclusion, the current study indicates a significant impact of UFR on the production of regulatory and proinflammatory monokines in patients under polysulphone haemodialfiltration. UFR reduction resulted in increased IL-10, but decreased IL-1$\beta$ and IL-6 production upon LPS stimulation. Since it was shown that monokine production correlates closely with the in vivo immune status in cuprophane haemodialysis, further studies are necessary to understand fully the pathophysiological mechanism and investigate the clinical relevance for patients under polysulphone haemodialfiltration.

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