Research

Effect of hemofiltration filter adsorption on circulating IL-6 levels in septic rats

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Abstract

Introduction Hemofiltration may modulate the inflammatory response in sepsis through a variety of mechanisms. We sought to distinguish clearance from adsorption as the principal mechanism responsible for reducing circulating IL-6 levels with hemofiltration.

Materials and methods Nine hours after cecal ligation and puncture in 18 adult male Sprague–Dawley rats, we divided the rats into three groups (6 animals each) and placed groups 2 and 3 on a hemofiltration circuit connected between the right carotid artery and femoral vein using an AN69 membrane. In the hemofiltration group (group 2), ultrafiltrate was replaced with lactated Ringer's solution; in the recirculation group (group 3), the ultrafiltrate was reinfused into the animal. A sham group (group 1) had an arteriovenous circuit inserted but no hemofiltration. Blood was obtained for measurement of IL-6 and tumor necrosis factor (TNF) at the start of hemofiltration and after 5 and 11 hours of treatment.

Results and discussion IL-6 levels increased only in the sham-treated animals (20.4 ± 11.3 at baseline to 62.3 ± 16.8 pg/ml at 11 hours, $P = 0.03$) (differences between groups 1 and 2, $P = 0.015$, and groups 1 and 3, $P = 0.028$). TNF levels were highly variable but not significantly different among the three groups.

Conclusion Hemofiltration-associated reductions in circulating IL-6 levels appear to be secondary to adsorption of mediators to the filter membrane. We do not know whether this is due to direct adsorption of IL-6 per se or to the absorption of other mediators with secondary downregulation of IL-6 production or release. In addition, we could not exclude an interaction between adsorption and hemofiltration.

Keywords continuous renal replacement therapy, cytokines, hemofiltration, interleukins, sepsis, tumor necrosis factor

Introduction

Numerous studies have shown that hemofiltration using porous synthetic membranes can and does remove a wide range of substances that mediate inflammation from the plasma [1–2]. Limited evidence supports the notion that this treatment can also influence circulating plasma concentrations of various mediators [3–5] and mounting evidence suggests that these manipulations have important biologic effects [6–8]. Despite these advances, there are no randomized clinical trials demonstrating that hemofiltration improves outcome in patients with sepsis. Furthermore, the exact mechanisms responsible for immunomodulation with hemofiltration remain uncertain. Modern hemofiltration filters may adsorb mediator substances as well as permitting them to pass through the membrane. Determining which of these mechanisms is dominant will be essential to advancing the design of materials and methodology suited to this form of therapy. If adsorption is the primary effect, the surface area of the filters must be

ANOVA = one-way analysis of variance; AUC = area under the curve; CLP = cecal ligation and puncture; ELISA = enzyme-linked immunosorbent assay; IL = interleukin; SNK = Student–Newman–Keuls; TNF = tumor necrosis factor.

increased, perhaps by using beads rather than filaments. Conversely, if mediator substances are removed mainly by sieving, increases in ultrafiltration rates will be required.

We undertook these experiments to determine whether adsorption alone could affect the inflammatory state of rats with sepsis in a manner similar to standard hemofiltration. We chose to measure interleukin-6 (IL-6) as a marker of the activation status of the cytokine network because it is known to persist for several hours in the circulation and reflects the influences of several other cytokines [9–10]. Although IL-6 is not cytotoxic, circulating IL-6 is reproducibly detectable in patients with sepsis, and higher concentrations portend a poor outcome [11]. For these reasons, IL-6 has increasingly been used a marker of the inflammatory response in clinical trials of investigational agents in sepsis [12]. We also measured tumor necrosis factor (TNF) in an effort to correlate changes in IL-6 with TNF activity during the same period.

Materials and methods

Surgical preparation

After approval by the Animal Care and Use Committee of the University of Pittsburgh Medical Center, we anesthetized 20 adult, male Sprague–Dawley rats (mean weight 486 g) with pentobarbital sodium (50 mg/kg intraperitoneally). We performed a midline laparotomy, exteriorized the cecum, and placed a ligature inferior to the ileocecal valve using 4-0 silk. We punctured the cecum three times using a sterile 18-gauge needle, placing one puncture site on each of the three antimesenteric surfaces. We then returned the cecum to the abdominal cavity and closed with 2-0 suture. We then administered a subcutaneous bolus of saline (50 ml/kg) as fluid resuscitation and returned the animals to their cages and allowed food and water ad libitum. In two animals, we obtained serial blood samples each hour for 24 hours and measured IL-6 and TNF concentrations. The results from these animals were used to plan the experiments for the remaining groups. We randomized the remaining 18 rats into three groups. Seven hours after the cecal ligation and puncture (CLP), we reanesthetized each animal and intubated it with a beveled, 16-gauge angiocatheter and ventilated it with room air using a Harvard rodent ventilator (Holliston, MA, USA) at a tidal volume of 10 ml/kg and a frequency sufficient to maintain an arterial PCO_2 between 35 and 45 mmHg. We isolated the right carotid artery and the left femoral vein by dissection and cannulated each with 1.27-mm PE-90 tubing. The tubing was formed into a catheter by inserting a beveled 20-gauge needle into one end. We flushed each cannula with heparinized saline (2000 units/liter). We placed a 3-way stopcock on each catheter so that blood sampling could be achieved using the carotid arterial catheter and fluid could be administered through the venous catheter.

Experimental procedure

Nine hours after CLP, we connected the arterial and venous catheters for animals in groups 2 and 3 to a clamped hemofilter with an AN-69 membrane (Miniflow-10, Hospal, Lyon, France), surface area = 0.042 m^2, fiber internal diameter 240 μm, fiber wall thickness 50 μm. The filter, which had been flushed with sterile saline only, was flushed with 5000 unit/liter heparinized saline immediately before the circuit was opened.

For animals in group 1, we connected the arterial and venous cannulas to each other and we removed 3 ml of blood to control for the deadspace of the filter, and replaced this volume with heparinized saline. The circuits were driven by the arterial blood pressure. The replacement fluid consisted of the following: 1:1 replacement of blood with 6% Hetastarch in saline, and 1:1 replacement of ultrafiltrate production with lactated Ringer's solution. We infused the lactated Ringer's solution as predilution into the arterial limb of the hemofilter. We gave the Hetastarch through the catheter in the femoral vein. We collected the ultrafiltrate and measured it twice an hour to maintain an accurate replacement rate (average 30 ml/hour). We began replacing the ultrafiltrate after the first half-hour.

For animals in group 3, we reinfused ultrafiltrate as replacement fluid. We chose to study reinfusion rather than ultrafiltrate clamping because sieving may augment adsorption by increasing the effective surface area of the membrane by exposing more of the inner matrix of the filter to plasma.

Blood was drawn from all the animals at hours 9, 14 and 20. The initial blood sample included blood for blood gas analysis (1.0 ml total). All other blood samples were 0.75 ml. We measured the hemoglobin concentration and oxygen saturation in each blood sample using an OSM3 Hemoximeter (Radiometer America, Inc, Westlake, Ohio, USA) to ensure that the animal was neither hypovolemic nor hypoxic. The blood sample was divided into two 0.4-ml chilled tubes containing EDTA (1 mg/ml) and centrifuged immediately at 1000 ***g*** for 10 minutes. The plasma was drawn off, placed into 0.5-ml labeled microtubes and stored at −70°C for later cytokine analysis. The animals remained anesthetized and on the ventilator until the 20th hour after the laparotomy, when we killed them humanely.

Measurements and calculations

Plasma concentrations of IL-6 and TNF were determined by rat-specific ELISA according to manufacturer instructions (Biosource International, Camarillo, CA, USA, and Endogen, Woburn, MA, USA), respectively. The TNF assay was linear from 0 to 2500 pg/ml, and the IL-6 assay was linear from 0 to 2000 pg/ml; coefficients of variation on repeated samples were less than 10% for both assays.

Our primary analysis was based on the change in IL-6 concentrations from the beginning to the end of the hemofiltration (9 hours versus 20 hours) across the three groups. The absolute changes in IL-6 concentrations were compared

using one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls (SNK) multiple comparison test. Our secondary analysis of TNF concentrations was performed after calculating the area under the curve (AUC) formed by three measurements (at 9, 14 and 20 hours) and zeroed by the baseline (9 hours) levels. Mean areas were compared for each group using ANOVA/SNK analysis as per IL-6 above. The absolute changes in IL-6 were correlated across all groups with the TNF AUC, using linear regression. All statistical tests were performed using MedCalc® (v 4.2, Mariakerke, Belgium) software and statistical significance was assumed for $P < 0.05$. Unless specified otherwise, all results are presented as means ± standard deviation.

Results

In the two animals used for mapping IL-6 and TNF responses to CLP, we found results consistent with those of other investigators [13]. Namely, plasma IL-6 concentrations rose steadily, peaking between 18 and 20 hours after CLP, whereas no consistent pattern emerged for plasma TNF levels. The results for the three groups are shown in Table 1. IL-6 levels were not significantly different among the three groups at baseline and at 5 hours. However, IL-6 levels increased in the sham-treated animals (group 1) from baseline to 11 hours (see Table 1; $P = 0.03$) (differences between groups 1 and 2, $P = 0.015$ and groups 1 and 3, $P = 0.028$) (Fig. 1). TNF levels were highly variable between and within animals. TNF AUC analysis was not significantly different among the three groups, although the trend was in the same direction as for IL-6, in that group 1 tended to have the highest TNF concentrations (453 ± 509 pg/ml per 11 hours), group 2 the lowest (160 ± 443 pg/ml per 11 hours) and group 3 intermediate (359 ± 377 pg/ml per 11 hours). The relation between the change in IL-6 and the TNF AUC is shown in Fig. 2. The weak correlation between the two as shown by linear regression was not statistically significant.

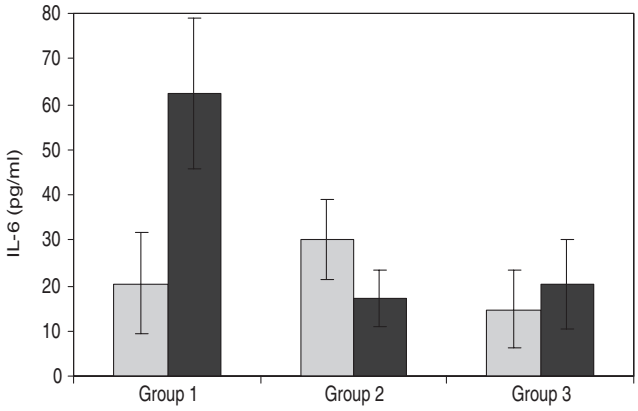
Discussion and conclusion

Our findings show that the inflammatory response induced by CLP and characterized by a steady increase in circulating IL-6 levels over 18–24 hours can be inhibited to a similar degree by hemofiltration whether or not the ultrafiltrate is reinfused. These findings provide strong evidence that immunomodulation seen with hemofiltration is predominantly the result of adsorption of circulating mediator substances to the filter membrane. Future studies of hemofiltration in sepsis might be more successful if steps are taken to maximize adsorption.

Our findings also confirm those of our previous trial in humans [3] and work from other groups [4–5] showing that biologically significant immunomodulation can and does occur with continuous hemofiltration. However, the clinical significance of these changes remain in question. Unlike therapies designed to target specific mediators, hemofiltration has the theoretical advantage of being at once selective and nonspecific [14]. Hemofiltration is selective in the sense that it will affect circulating substances in direct proportion to their concentrations in the plasma, but nonspecific in that it will remove multiple substances, both proinflammatory and anti-inflammatory [1–2]. This feature makes hemofiltration quite different from other forms of immunomodulation that target specific mediators and reduce their concentration regardless of the baseline levels.

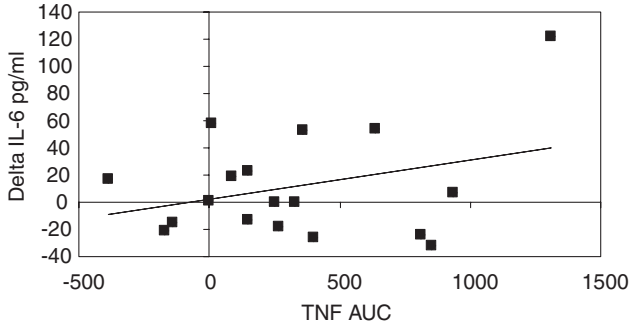
Hemofiltration has been shown remove a wide variety of biologically active substances, many of which are known to be involved in the regulation of host defense, inflammation and

Figure 1



Plasma IL-6 concentrations in rats before (light bars) and after (dark bars) 11 hours of hemofiltration started 9 hours after cecal ligation and puncture (CLP). Animals in group 1 underwent CLP with sham hemofiltration. Animals in group 2 underwent CLP and hemofiltration. Animals in group 3 underwent CLP and hemofiltration with reinfusion of the ultrafiltrate intravenously. Heights of bars correspond to mean IL-6 levels; error bars = SEM. Significant differences were found for group 1 before versus after, and after hemofiltration between groups 1 and 2 and 1 and 3 ($P < 0.05$). SEM, standard error of the mean.

Figure 2



Scatter plot of tumor necrosis factor area-under-the-curve (TNF AUC) versus the change in IL-6 before and after hemofiltration in rats that had undergone cecal ligation and puncture. The regression line was determined by linear regression ($y = 0.0289x + 2.1158$), $r = 0.3217$ (95% confidence interval for $r = -0.1709$ to 0.6856).

Table 1

Cytokine concentrations (pg/ml; mean ± SEM) found in rats after cecal ligation and puncture followed by hemofiltration

	Group 1[a]		Group 2[b]		Group 3[c]	
Time (h)	IL-6	TNF	IL-6	TNF	IL-6	TNF
0	20.4 ± 11.3	7.6 ± 7.6	29.9 ± 8.9	65.7 ± 23.7	14.7 ± 8.6	41.1 ± 22.4
5	32.1 ± 8.9	54.7 ± 23.8	34.4 ± 10.1	105.2 ± 36.6	21.1 ± 11.2	79.3 ± 25.6
11	62.3 ± 16.8*	63.1 ± 23.0*	17.1 ± 6.3#	49.3 ± 18.2	20.2 ± 9.9#	83.6 ± 29.8*

[a]Sham group: arteriovenous circuit inserted but no hemofiltration. [b]Ultrafiltrate replaced with lactated Ringer's solution. [c]Ultrafiltrate reinfused into the animal. *$P < 0.05$ in comparison with baseline (0 h). #$P < 0.05$ in comparison with group 1. SEM, standard error of the mean; TNF, tumor necrosis factor.

tissue repair. However, controversy exists as to the primary mechanism responsible for this effect. We have previously shown, in patients with sepsis, that time-weighted mean TNF concentrations decreased during continuous venovenous hemofiltration but increased if the same patients were treated with continuous venovenous hemodialysis [3]. Despite these changes in circulating TNF levels, only trace amounts of TNF were recovered from the ultrafiltrate. Subsequently, other groups have found similar results in patients treated with continuous venovenous hemofiltration on cardiopulmonary bypass [4] or in patients with septic shock [5]. However, both of these recent studies showed only early, transient reductions in plasma cytokines, suggesting that filter saturation occurs after several hours of therapy. The various membranes currently available differ significantly from one another in their adsorptive capacity for mediators [15]. Indeed, the majority of studies of hemofiltration in sepsis have failed to find any change in circulating mediator concentrations. These observations have led to the development of combined hemofiltration/hemoadsorption techniques, which are beginning to show promise in animal studies [16].

Our results do not exclude a possible role for mechanisms other than adsorption. Several investigators have found an association between increased ultrafiltration rates and improvement in hemodynamic stability in both animals and humans with septic shock [5–8]. It may be that these improvements in hemodynamic stability do not involve cytokine removal. Furthermore, filtration appears to augment adsorption [3], so that combined filtration/adsorption might be more effective than adsorption alone, at least when hollow-fiber dialyzers are used. Other methods of increasing adsorption might be even more effective [17].

A major limitation to our study is the fact that we cannot distinguish between increased IL-6 removal and decreased IL-6 production as the cause for the observed attenuation in IL-6 response to CLP. We chose to use IL-6 as our marker of the inflammatory response because, unlike many other cytokines, its plasma concentrations have been directly correlated with risk of death in intra-abdominal sepsis in humans, and measurements of this single parameter predicted outcome in these cases with remarkable (82.9%) accuracy [18]. Furthermore, although IL-6 is not directly cytotoxic, there is evidence that it modulates other inflammatory cytokines [19–20], and plasma concentrations have been found to be 69% (95% confidence interval 30%–108%) higher in nonsurvivors than in survivors with sepsis [9]. Many studies have shown an association between mean plasma IL-6 concentrations over time and mortality [21], and persistent elevations in IL-6 appear to be more important than initial or peak levels [10]. However, plasma IL-6 levels reflect both clearance (endogenous and exogenous) and production, and IL-6 production is largely determined by the activity of other proinflammatory cytokines (especially IL-1 and TNF).

Thus, hemofiltration may attenuate the sepsis-induced rise in IL-6 by adsorption of IL-6 or by adsorption of other mediators or both. Indeed, it seems likely that our findings resulted from a downregulation in the inflammatory response to CLP. This explanation is speculative, however, because we could not demonstrate a corresponding decrease in plasma TNF. Analysis of plasma TNF concentrations is more complex than of IL-6, because the TNF response is already waning by 9 hours after CLP [22] and is much more variable. Furthermore,

Key messages

- Changes in IL-6 seen with hemofiltration are predominantly the result of adsorption of circulating inflammatory mediator substances to the filter membrane
- Biologically significant immunomodulation can and does occur with continuous hemofiltration at blood flow rates currently in use in clinical practice
- We cannot distinguish between increased IL-6 removal and decreased IL-6 production as the cause for the attenuation in the IL-6 response to sepsis observed with hemofiltration
- Future studies of hemofiltration in sepsis might be more successful if steps are taken to maximize adsorption

we measured total TNF and do not know the proportion that was biologically active at the time. We chose to analyze TNF using AUC because of these concerns and because the effects of TNF over time presumably relate to outcome and to IL-6 expression more than to a concentration at a single time point. Unfortunately, the smallness of the animals precluded multiple sampling and we selected only three time points in order to obtain the 'curve' used for AUC analysis. These limitations and the small sample size perhaps explain why we were only able to show a weak correlation between TNF AUC and changes in IL-6 concentrations over time.

Finally, we must acknowledge that our animal model of sepsis, like sepsis in humans, is associated with a variable inflammatory response (Table 1). However, both absolute and relative changes in IL-6 levels appear similar with hemofiltration with or without recirculation of ultrafiltrate. These results suggest that adsorption is the primary, though perhaps not the sole, mechanism responsible for changes in IL-6 levels during hemofiltration.

Competing interests

None declared.

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