Alterations in Soluble and Leukocyte Surface L-Selectin (CD 62L) in Hemodialysis Patients[1,2]

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ABSTRACT

Hemodialysis (HD) patients can develop acute reactions during treatment as well as increased long-term susceptibility to infections and malignancy. Leukocyte-membrane interactions may contribute to these processes. The effects of a single HD session on L-selectin, a leukocyte adhesion molecule for endothelium, were examined. Serum levels of soluble L-selectin were measured in 23 patients by enzyme-linked immunosorbent assay before and after a 3-h dialysis session. There was a statistically significant increase in soluble L-selectin from 1.36 ± 0.12 (SE) to 1.57 ± 0.18 μg/mL ($P < 0.01$). An increase in shed L-selectin was observed for the "venous" compared with the "arterial" port of the dialyser ($P < 0.01$) 15 min into HD. Soluble L-selectin levels were found to remain increased at 3 h after treatment. Leukocyte-bound L-selectin and CD11b was examined by the use of flow cytometry. Neutrophil L-selectin decreased to 69 ± 7% at 15 min ($P < 0.01$) and then rebounded to 98 ± 7% at 180 min. Monocyte and lymphocyte L-selectin did not decrease. Because L-selectin is important for leukocyte attachment to endothelium at sites of inflammation, alterations of shed L-selectin and cell-surface L-selectin levels may play a role in the immunologic consequences of HD treatment.

Key Words: *Uremia, membrane, adhesion, receptors, immunodeficiency*

Patients on chronic hemodialysis (HD) have a propensity to develop infections and have an increased incidence of malignancy (1,2). Uremic patients also have clinical evidence of impaired lymphocyte function, such as measured by prolonged skin graft survival, decreased skin test reactivity, and an impaired response to hepatitis B vaccination (3–5). These defects in immunity result from the uremic state or are a consequence of therapy. Hemodialysis is associated with complement activation (6), up-regulated expression of granulocyte cell surface integrins (7), and the release of proinflammatory cytokines (8). Leukocyte-membrane interactions have been implicated as being responsible for some of the clinical problems observed in HD patients such as dialysis-related hypoxia (9) and arthropathy from β-2 microglobulinemia (10). Leukocyte-membrane interactions are mediated in part by leukocyte adhesion molecules, molecules that normally play key physiologic roles in cell-cell and cell-matrix interactions.

The leukocyte adhesion molecule CD11b/CD18 has been shown to be up-regulated in HD and may play a role in the pulmonary sequestration of granulocytes during HD (7,11). To date, most studies on leukocyte adhesion in HD have looked at the integrin family of adhesion receptors; however, other families of adhesion molecules are rapidly being discovered. The selectins are a family of leukocyte adhesion molecules characterized by a unique structure including an amino-terminal C-type lectin domain that binds carbohydrate-containing ligands (12,13). There are three known members (L, E, P), each named for the initial cell type with which they were found to be associated (leukocyte, endothelium, platelet). L-Selectin is expressed on most leukocytes and appears to mediate lymphocyte binding to high endothelial venules of peripheral lymph nodes, as well as lymphocyte, neutrophil, and monocyte attachment to endothelium at areas of inflammation (12,14). L-Selectin can be shed from the leukocyte surface after cellular activation (15). This shed form of L-selectin (sL-selectin) retains functional activity and may modulate leukocyte binding to endothelium *in vivo* (16). Increasingly, it is felt that L-selectin may play a role in select normal and abnormal immune responses.

In view of the recognized clinical immunodeficiency seen in HD patients, as well as the cellular activation induced during HD, alterations in L-selectin may be important. The effects of HD on leukocyte-bound L-selectin have been well studied by Himmelfarb *et al.*, who postulated that L-selectin shedding may occur during dialysis (11). With the recent development of a method to assess the shed L-selectin receptors, we

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examined the effects of HD on SL-selectin in serum. Soluble L-selectin levels were quantitated with enzyme-linked immunosorbent assay (ELISA) and compared with levels leukocyte-bound L-selectin and CD11b.

METHODS

Subjects

Twenty-three men with ESRD on chronic HD were studied at the James Haley VA Hospital and the University of South Florida Dialysis Center. Patients ranged in age from 24 to 77 (mean, 53). Chronic renal failure was due to diabetes in 10 cases, hypertension in 6 cases, glomerulonephritis in 3 cases, interstitial nephritis in 2 cases, nephrectomy of solitary kidney in 1 case, and polycystic kidney disease in 1 case. Patients underwent HD thrice weekly, 3 to 4 h each per session, with bicarbonate-based dialysate and heparin anticoagulation. All patients were dialyzed to a constant target ultrafiltration pressure to attain dry weight. New cuprophane membranes were used with no dialyzer reuse. Study patients had no active infections, obvious malignancies, or inflammatory conditions and were not taking immunosuppressive medications.

Blood samples were taken from the dialysis patients through the existing vascular access. The study was approved by the Institutional Review Board of the University of South Florida and the Research and Development Committee of the James Haley Veterans Hospital. Informed consent was obtained from each patient.

SL-Selectin ELISA

Three-milliliter samples of blood were obtained from the subjects, and serum samples were stored at −70°C until assayed for soluble L-selectin. The ELISA used to quantitiate SL-selectin has been described in detail elsewhere (17). Briefly, 96-well microtiter plates (Costar, Cambridge, MA) were coated with an anti-L-selectin monoclonal antibody (mAb) blocked with 2% bovine serum albumin and 1% gelatin. Serum samples (50 μL) were added to triplicate wells. Each assay included a previously quantified plasma sample used to generate a standard curve. Plates were then incubated with biotinylated anti-LAM1–3 mAb for 60 min. The plates were washed, and avidin-horseradish peroxidase (Pierce Chemical Co., Rockford, IL) was added for 30 min. Plates were developed with *o*-phenylenediamine (Sigma Chemical Co., St. Louis, MO) in 0.1 M citrate buffer in the presence of H_2O_2. The OD of each assay well was quantified with an ELISA reader, and values are presented as the mean of triplicate wells.

Cellular Preparations

Heparinized blood samples were processed for flow cytometry by use of the following procedure. A total of 100 μL of freshly collected whole blood and 10 μL of antibody were gently mixed together. Fluorescein isothiocyanate-labeled mAb to L-selectin was kindly provided by Coulter Immunology (Hialeah, FL). PE-labeled anti-CD11b and CD14, and fluorescein isothiocyanate-labeled CD45 and isotype-matched control mAb were obtained from Becton Dickinson Immunology Systems (San Jose, CA). The mixtures were incubated in the dark at room temperature for 30 min, and then 2 mL of lysing reagent (Becton Dickinson, San Jose, CA) was added to each tube. The samples were incubated for another 10 min in the dark, and the cells were pelleted and resuspended in phosphate-buffered saline (Sigma). After another 5-min centrifugation at 1,200 rpm, the supernatant was removed and the cells were resuspended in 0.5% formalin in phosphate-buffered saline at 4°C for 30 min before flow cytometry.

Two-Color Flow Cytometry

Samples were analyzed with Simulset Software (Becton Dickinson, San Jose, CA) on a FACScan flow cytometer (Becton Dickinson). A minimum of 22,000 cells were collected for each sample. Granulocyte, monocyte, and lymphocyte gate purity was assessed by forward light scatter, side light scatter, CD45 staining, and CD14 staining. Data were saved in list mode. Nonspecific fluorescence staining was assessed for each sample and cell type with isotype-specific control antibodies. The mean channel fluorescence was determined for CD11b and L-selectin for each sample and each cell type after reanalysis with PC-Lysys Software (Becton Dickinson).

Statistical Analysis

Paired *t* tests were used to compare matched values, and analysis of variance was used for comparing different groups. All values are expressed as mean ± SE, and the differences were considered to be significant when $P < 0.05$.

RESULTS

Effect of Hemodialysis Treatment on Soluble L-Selectin Levels

In 23 subjects, pre-HD SL-selectin levels were 1.36 ± 0.12 μg/mL and then increased after the HD treatment to 1.57 ± 0.18 μg/mL ($P < 0.01$) (Figure 1). Pre-HD and post-HD samples were obtained during the same session.

Simultaneous blood sampling of "arterial" (inflow) and "venous" (outflow) ports at 15 min into HD was performed on 23 patients at a different collection date than the "pre" and "post" sampling. SL-Selectin was significantly higher on the "venous" side (1.22 ± 0.12 *versus* 0.97 ± 0.11 μg/mL; $P < 0.01$) (Figure 2).

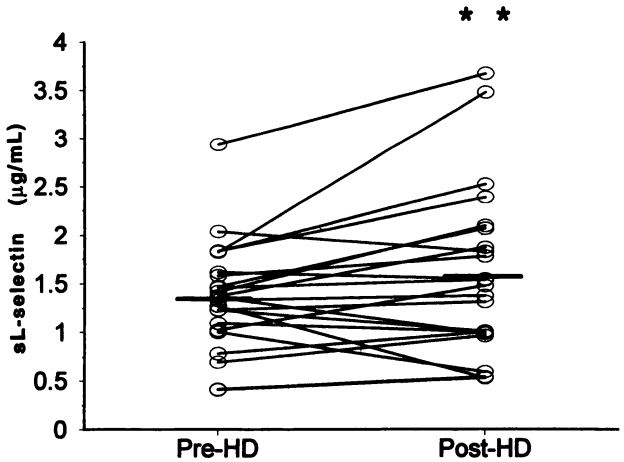

Figure 1. The effect of a 3-h HD session on SL-selectin ($N = 22$). There was a significant increase in SL-selectin at the end of HD. $^{**}P < 0.01$.

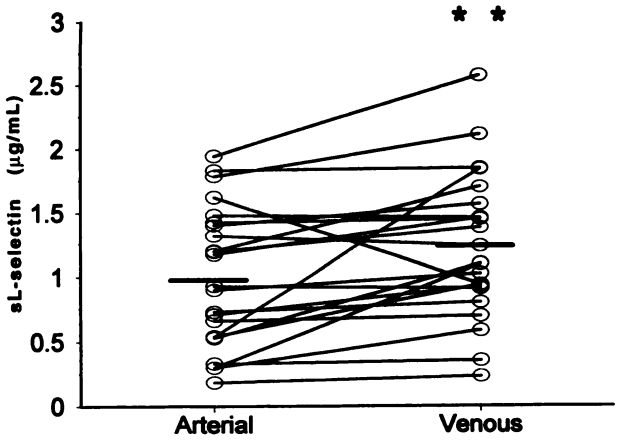

Figure 2. Simultaneous measurement of sL-selectin from the "arterial" and "venous" ports of the dialyzer at 15 min into HD ($N = 23$). There was a significant increase in the sL-selectin in the "venous" port of the dialyzer. *$P < 0.01$.

The kinetics of sL-selectin were evaluated during and after HD in eight patients. The increase in sL-selectin became statistically significant at 3 h into HD ($P < 0.05$). sL-Selectin remained elevated compared with pre-HD levels at 1 h ($P < 0.01$) and 3 h ($P < 0.01$) after the end of the HD treatment (Figure 3).

Leukocyte-Bound L-Selectin Levels

Flow cytometric analysis on 10 patients revealed that granulocyte-bound L-selectin decreased within 15 min of HD (69 ± 7%; $P < 0.01$) before returning to baseline at 180 min of HD (Figure 4a). Monocyte L-selectin increased within 15 min of HD (140 ± 7%; $P < 0.01$) and then returned to close to baseline, but was still elevated (114 ± 5%; $P < 0.05$), at 180 min of

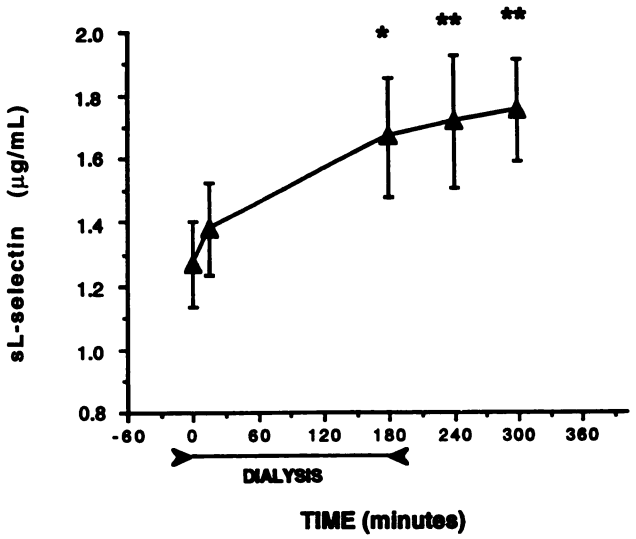

Figure 3. Time course of sL-selectin during HD ($N = 8$). sL-Selectin stayed elevated at 1 and 3 h after the end of HD. *$P < 0.05$, **$P < 0.01$.

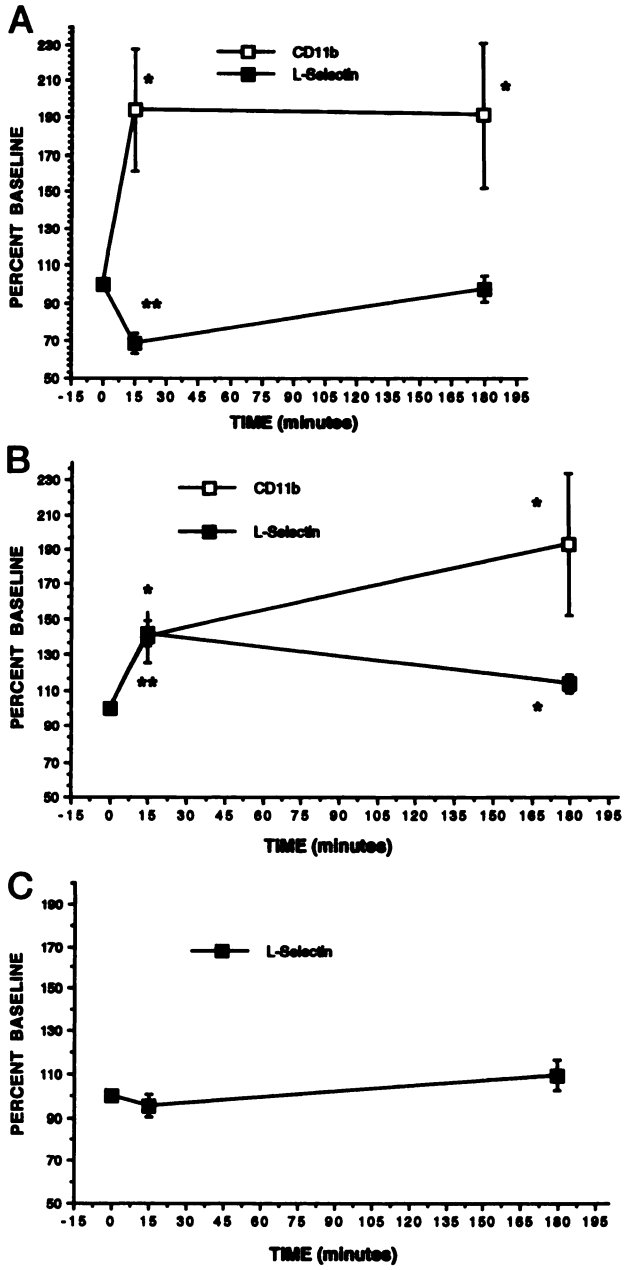

Figure 4. Leukocyte-bound L-selectin and CD11b as determined by flow cytometry ($N = 10$). (a) Neutrophil L-selectin decreased at 15 min into HD and then rebounded to baseline by the end of HD. CD11b rose at 15 min and remained elevated at the end of HD. (b) Monocyte L-selectin increased at 15 min and then decreased close to baseline (but was still elevated) at the end of HD. Monocyte CD11b increased at 15 min and continued to rise by the end of HD. (c) Lymphocyte L-selectin stayed constant through HD. Lymphocyte CD11b was not detected. *$P < 0.05$, **$P < 0.01$.

HD (Figure 4b). Lymphocyte L-selectin did not change with the HD treatment (Figure 4c).

Neutrophil-bound CD11b increased within 15 min

of HD (194 ± 33%; $P < 0.05$) and remained elevated at 180 min of HD (191 ± 40%; $P < 0.05$). Monocyte-bound CD11b increased within 15 min of HD (140 ± 15; $P < 0.05$) and then continued to increase by 180 min of HD (194 ± 40%; $P < 0.05$).

DISCUSSION

These studies demonstrate that serum soluble L-selectin increases after 3 h of dialysis treatment and stays elevated for at least another 3 h after the end of dialysis. At 15 min into HD, SL-selectin levels are elevated in the outflow tract of the dialyzer compared with the inflow, suggesting an effect caused by the dialysis apparatus. The rise in SL-selectin is associated with a decrease in neutrophil-bound L-selectin but not lymphocyte L-selectin. Conversely, monocyte surface L-selectin rose during the HD treatment.

The physiologic role of receptor shedding is unknown, but it is attractive to speculate that shedding after ligand binding is a way of deactivating the receptor. For a locomoting leukocyte, this action may allow for deadhesion after adhesion. *In vitro*, various cell-activating agents, including phorbol esters, can result in the shedding of L-selectin (18–21). A host of cytokines are released into the circulation during HD (22), and it is well established that granulocyte and monocyte activation occurs during treatment (8,23). However, the occurrence of lymphocyte activation by HD is being debated (24–26).

After observing the rise in SL-selectin during the HD treatment, we sought to identify the cell type from which it was most likely shed. Our finding of a drop in granulocyte-bound L-selectin at 15 min into HD suggests that this was the most likely source of the shed receptor. The rise in monocyte-associated L-selectin is difficult to interpret, although it is probable that an increase in mobilization to the surface outweighed receptor shedding. Although lymphocytes have been shown to shed L-selectin on activation, we found no change in the level of lymphocyte-bound L-selectin after HD. Our results on neutrophil and lymphocyte surface L-selectin confirm those of Himmelfarb *et al.*, who also observed a decrease in granulocyte-bound, but not lymphocyte-bound, L-selectin during HD (11). They postulated, as we have now demonstrated, the occurrence of L-selectin shedding associated with the decrease on the neutrophil surface. Our study patients were all dialyzed with new cuprophane membranes, and Himmelfarb *et al.* observed similar effects on neutrophil- and lymphocyte-bound L-selectin with new cellulose membranes. Interestingly, but perhaps not unexpectedly, they did not see the decrease in granulocyte L-selectin when they used "reused" dialysis membranes (which may cause less leukocyte activation). Because of current regulations on using only new membranes in the dialysis units from which subjects were recruited, we did not evaluate the effects of "reuse" on SL-selectin. Future studies evaluating the effects of more biocompatible and high-flux membranes on SL-selectin shedding would also be useful. It is also important to note that the interpretation of changes in leukocyte receptors during HD must take into account that leukocyte populations (particularly neutrophils) studied during early dialysis are removed from the circulation and may be different from leukocytes collected and studied at a later time.

The early part of HD treatment is associated with systemic neutropenia, pulmonary leukoaggregation, and neutrophil activation (11). We evaluated simultaneous "arterial" and "venous" SL-selectin levels at 15 min into HD at a separate time from the "pre" and "post" sampling and found a significant increase in the SL-selectin in the effluent of the dialyzer returning to the patient. Hence, it is likely that leukocyte-membrane interactions provided the stimuli for L-selectin shedding. Although this bioincompatibility process is associated with the production of many different inflammatory mediators and cytokines, which could lead to neutrophil activation and subsequent shedding of the L-selectin, complement adherence and activation by the membrane have been postulated to be one of the initiating steps (11).

Shedding of the L-selectin may have a number of physiologic effects in the HD patient. With the progressive increase in CD11b expression by the neutrophil, the shedding of L-selectin may explain why the granulocytes no longer become sequestered in sites like the lung and why the initial neutropenia resolves by the end of HD (11). This shedding may be a valuable mechanism that prevents the activated neutrophil in HD from damaging normal tissue (27). However, there may be a dual effect and the shed L-selectin may also be deleterious in this setting. We observed that the SL-selectin remained elevated for at least 3 h after HD. It is possible that these "high CD11b, low L-selectin" granulocytes are less effective in combating infections. This is important because infection is one of the leading causes of morbidity and mortality in HD patients.

Recently, low levels of plasma SL-selectin have been associated with the development of adult respiratory distress syndrome and increased morbidity and mortality in critically ill patients (28). In this context, it has been speculated that panendothelial activation, felt to be important in the pathogenesis of adult respiratory distress syndrome, may result in the sequestration of SL-selectin in microvascular beds (28).

With the increase in SL-selectin, we also studied leukocyte-bound CD11b for comparison, and our results were similar to those of previously published reports: a rise at 15 min into HD in granulocyte CD11b that remained elevated at 180 min, whereas monocyte CD11b rose at 15 min and was further elevated at 180 min (7,11,23). An increase in serum proteins can be seen at the end of HD, reflecting hemoconcentration due to fluid removal (H. Rabb, unpublished results). We cannot rule out the possibility that, in part, the rise in SL-selectin was due to hemoconcentration. Although difficult to quantify ex-

actly, one can estimate that 2 to 3 L of 40 L of body water was removed per treatment. Thus, a 5 to 7% increase in the concentration of nondialyzed proteins could occur. However, Kaupke *et al.* have found no increase in soluble intracellular adhesion molecule-1 after HD treatments (29). Another aspect of our study is that we evaluated both diabetics and nondiabetics. Diabetes has been associated with abnormal polymorphonuclear leukocyte function (30). Therefore, we compared the sL-selectin data from the diabetic with the nondiabetic patients in our study (data not shown), and there was no difference between the two groups.

This study is the first demonstration of changes in sL-selectin during HD. With the rapid developments in leukocyte biology, these and many more abnormalities in soluble adhesion molecules are likely to be found in the uremic patient. Although high levels (8 to 15 μg/mL) of sL-selectin can block normal leukocyte adhesion *in vitro* (16), its physiologic significance is unknown. Further studies on the effects of different dialysis membranes on soluble L-selectin as well as *in vitro* studies to understand the pathophysiology of these changes in HD may help elucidate some of the mechanisms of the immunodeficiency observed in the patient on chronic HD. The HD population is growing, with over 130,000 patients in the United States alone (31). Despite our success in maintaining life, we have yet to make major inroads in improving the morbidity and functional levels in this population. Efforts to understand and treat factors leading to immunologically related morbidity would have a major effect not only for the HD patients, but also for the health care network that supports them.

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