Diminished Chemokine and Cytokine-Induced Adhesion of CD4+T Cells to Extracellular Matrix Ligands in Patients with End-Stage Renal Failure

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Abstract

Background: Cell-mediated immunity is impaired in uremia. Cell-matrix interactions of immune cells such as CD4+T lymphocytes with extracellular matrix are an important requirement for an intact immune response. The adherence of CD4+T cells of healthy subjects (normal T cells) to ECM components is inhibited in the presence of uremic serum. Such decreased adhesive capacity is also found in T cells of dialysis patients. Various chemokines and cytokines affect the attachment of CD4+T cells to ECM.

Objective: To evaluate chemokine (MIP-1β and RANTES) and tumor necrosis factor-α-induced adhesion of CD4+T cells to ECM in a uremic milieu.

Methods: We examined adhesion of normal CD4+T cells (resting and activated) to intact ECM in response to soluble or bound chemokines (MIP-1β and RANTES) and to TNF-α following incubation in uremic versus normal serum. Thereafter, we evaluated the adhesion of resting CD4+T cells from dialysis patients in a similar fashion and compared it to that obtained from a healthy control group.

Results: Addition of uremic serum diminished soluble and anchored chemokine-induced attachment of normal resting and activated CD4+T cells to ECM compared to a normal milieu (a peak response of 10–11% vs. 24–29% for soluble chemokines, $P<0.001$; 12–13% vs. 37–39% for bound chemokines on resting cells, $P<0.01$; and 18–20% vs. 45–47% for bound chemokines on activated cells, $P<0.02$). The same pattern of response was noted following stimulation with immobilized TNF-α (7 vs. 12% for resting cells, $P<0.05$; 17 vs. 51% for activated cells, $P<0.01$). Adherence of dialysis patients' cells to ECM following stimulation with both bound chemokines was reduced compared to control T cells (15–17% vs. 25–32%, $P<0.0000$). In contrast, adherence following stimulation by TNF-α was of equal magnitude.

Conclusions: Abnormal adhesive capacity of T lymphocytes to ECM in uremia may, in part, be related to a diminished response to MIP-1β, RANTES and TNF-α. However, whereas reduced adhesion to chemokines was present in both normal CD4+T cells in a uremic environment and in dialysis patients' T cells, TNF-α-induced adhesion was found to be inhibited only in normal cells in a uremic milieu.

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Cellular immunity in end-stage renal failure patients is impaired [1,2]. This acquired immunodeficiency state may partly explain the high incidence of infections observed in these patients [3]. It has been shown that whereas the relative proportions of T cell subsets are unchanged [4], mitogenic lymphocyte responses are depressed [5]. The mechanisms responsible for the altered T cell function have not been fully elucidated. Immunosuppressive properties have been attributed to uremic serum [6–8].

The extracellular matrix is a complex network composed of glycosaminoglycans and adhesive glycoproteins, such as collagen, laminin and fibronectin. It appears that ECM serves not only as a supportive structure surrounding cells, but also as an integral network modulating cell activation, adhesion, growth and differentiation [9]. The ECM can thus regulate the migration of inflammatory immune cells, such as CD4+T lymphocytes. The recognition and ensuing interactions of CD4+T lymphocytes with ECM are mediated primarily by integrins of the b_1 subfamily and depend on the state of activation of the interacting T cells [10,11].

The CD4+T cell–ECM interactions are influenced by various cytokines, which are present as both soluble and bound mediators adjacent to sites of inflammation [12]. TNF-α markedly increases adhesion of CD4+T cells to ECM via protein tyrosine phosphorylation [13].

ECM = extracellular matrix
TNF-α = tumor necrosis factor-α

Chemokines, a group of cytokines responsible for chemotaxis, produce leucocyte migration via a two-step process. They first activate integrins, which mediate adhesion to endothelial cells and ECM, and thereafter promote the extrusion of adherent leukocytes across the endothelium and through the ECM [12]. Chemokines are divided into two major structurally distinct subfamilies that differ in their action on leukocyte subsets [12,14,15]. The chemokines MIP-1β (macrophage inflammatory protein) and RANTES (regulated on activation normal T cell expressed and secreted) stimulate CD4+T cell adhesion to ECM in an integrin-dependent manner [16]. Defects in cell-matrix adhesion may lead to an impaired immune response [12].

Following phorbol-12-myristate-13-acetate stimulation, uremic sera inhibit the adhesion of T cells from normal subjects to ECM, fibronectin and laminin in a dose-dependent fashion [17]. CD4+T cells from dialysis patients also exhibited abnormal adhesive activity [18]. The impaired function of these cells could be due to their inability to interact with ECM proteins in response to specific stimulation by chemokines and/or TNF-α. To evaluate this possibility, this current *in vitro* study was performed.

Patients and Methods
Patients
Blood was obtained by peripheral vein puncture from dialyzed patients (hemodialysis and continuous ambulatory peritoneal dialysis) and from normal subjects. Exclusion criteria included cachexia, diabetes mellitus, malignancies, recent infection, systemic disease (vasculitis, systemic lupus erythematosus, etc.), and immunosuppressive therapy. Hemodialysis was performed using bicarbonate as buffer. Blood samples were obtained just prior to a hemodialysis session. CAPD patients were dialyzed using DIALINE® peritoneal dialysis solutions (Travenol, Israel). All patients were considered to be well dialyzed during the 3 months prior to their participation in the study.

Study design
Evaluation of normal CD4+T cells. Adherence of normal CD4+T cells (resting and activated) to intact ECM in response to soluble or bound chemokines (MIP-1β and RANTES) or TNF-α was examined following incubation in serum obtained from 10 dialysis patients (hemodialysis and CAPD) versus 10 age- and gender-matched normal subjects (control group).

Evaluation of dialysis patients' CD4+T cells. Adhesion to ECM of resting CD4+T cells obtained from 26 dialysis patients (15 on hemodialysis and 11 on CAPD) was examined in response to bound chemokines (MIP-1β and RANTES) or TNF-α and compared to that of 13 age-

and gender-matched healthy volunteers (control group). The cells were incubated in normal serum.

Methods
Preparation of human CD4+T cells
CD4+T cells were purified from peripheral blood mononuclear leukocytes of dialysis patients and healthy donors. The mononuclear cells were isolated on a Ficoll gradient, washed, and incubated (37°C, 10% CO_2 humidified atmosphere) in tissue culture plates. After 2 hours, the non-adherent cells were removed and enriched for T cells by passage through nylon wool columns (Polysciences, Warrington, PA, USA). CD4+T cells were negatively selected using a mixture of anti-CD8, CD19 and CD14 mAb conjugated to magnetic beads (Advanced Magnetics, Cambridge, MA, USA). The resulting cell population consisted of over 90% CD3+/CD4+T cells.

Preparation of ECM-coated culture dishes
Flat-bottom microtiter wells were coated with ECM as previously described [19]. Briefly, freshly isolated bovine corneal endothelial cells ($5x10^4$cells/ml) were cultured in flat-bottom 96-well plates in DMEM supplemented with 5% dextran T-40, bovine calf serum, fibroblast growth factor (100 ng/ml), and antibiotics. After 6 to 8 days at 37°C in a 10% CO_2 humidified atmosphere, the confluent layers of endothelial cells were dissolved by exposure to phosphate-buffered saline/0.5% Triton X-100, 20 mM NH_4OH for 3 min at 22°C. This procedure yielded an intact ECM attached to the entire surface area of the wells that were free of serum proteins, nuclei, cytoskeletal and cellular debris.

Quantitation of CD4+T cell adhesion
The adhesion of CD4+T cells to intact ECM was assessed as previously described [20]. Briefly, CD4+T cells ($n=10^5$) of either normal subjects or dialysis patients, as indicated, were labeled (1 h, 37°C, 10% CO_2) with $Na_2^{51}CrO4$ (New England Nuclear, Boston, MA; 3–5 μCi/10^6 cells/100 μl of fetal calf serum), washed three times with PBS, counted, resuspended in adhesion medium (RPMI 1640 medium supplemented with 2% bovine serum albumin, 1 mM Ca^{2+}, 1 mM Mg^{2+}, 1% sodium pyruvate, and 1% HEPES) and added to the ECM pre-coated wells. Where indicated, PMA (25 ng/ml) was added to the wells for 30 min to activate the cells. Cytokines were dissolved in PBS at various concentrations, used as soluble mediators of cell adhesion by pre-incubation with the T cells (60 min at 37°C), and added to the ECM-coated wells along with the T cells. Bound mediators were added to the wells (1 ng/well for MIP-1β and RANTES and 20 ng/well for TNF-α, for 60 min at 37°C), unbound chemokines and TNF-α were removed by aspiration and

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CAPD = continuous ambulatory peritoneal dialysis

PBS = phosphate-buffered saline
PMA = phorbol-12-myristate-13-acetate

washing, and the wells were blocked with 1% BSA in PBS. After the addition of PMA and sera (normal or uremic), as indicated, to the labeled T cells in the coated wells, the plates were incubated (1 h, 37°C, 10% CO_2) and then washed three times with PBS to remove non-adherent cells. The adherent cells were lyzed with NaOH (1N) or Triton 0.1%, and the resulting supernatants were removed for counting. The amount (cpm) of [^{51}Cr] in the resulting supernatants corresponded to the percentage of bound cells. For each experimental group the results were expressed as the mean±SD percent of T cells bound in triplicate or quadruplicate wells. The percentage of bound cells per well was calculated as follows:

$$\frac{a/b}{c/b} \cdot 100$$

where:

a = cpm of residual cells in wells coated with ECM proteins

b = spontaneous release of ^{51}Cr from the added cells

c = total cpm of cells added.

Reagents

Recombinant human MIP-1β and RANTES generated in *Escherichia coli* were purchased from Pepro Tech Inc. (Rocky Hill, NJ, USA). Human rTNF-α was purchased from Genzyme (Cambridge, MA). Tissue culture dishes were obtained from Falcon Labware Division, Becton Dickinson (Oxnard, CA). Antibiotics, HEPES buffer, glucose, tissue culture media, calf sera and PBS were obtained from Biologic Industries (Beit HaEmek, Israel). Fibroblast growth factor was purchased from Sigma Chemical Co. (St. Louis, MO).

Statistical analysis

Significance was determined by using Students *t*-test for paired groups. $P < 0.05$ was considered to be statistically significant.
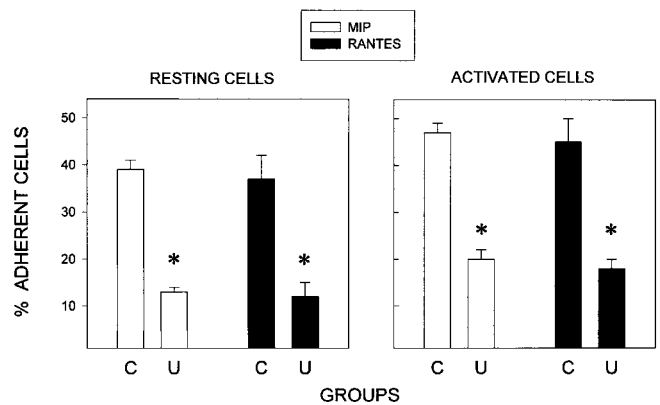
Results

Evaluation of normal CD4+T cells

- *Effect of uremic serum on induction of adhesion of resting CD4+T cells to ECM by diffusible chemokines* [Figure 1]. Resting CD4+T cells did not adhere to ECM — neither control (4±1%) nor patients' cells (3±1%). Soluble MIP-1β and RANTES induced adhesion of resting CD4+T cells to ECM in a bell-shaped dose-response pattern (concentrations of 0.001 to10 ng/ml with a peak response at 0.01 ng/ml). Addition of uremic serum diminished chemokine - induced adhesion of CD4+T cells, resulting in a blunted dose-response pattern (a peak response of 11

BSA = bovine serum albumin
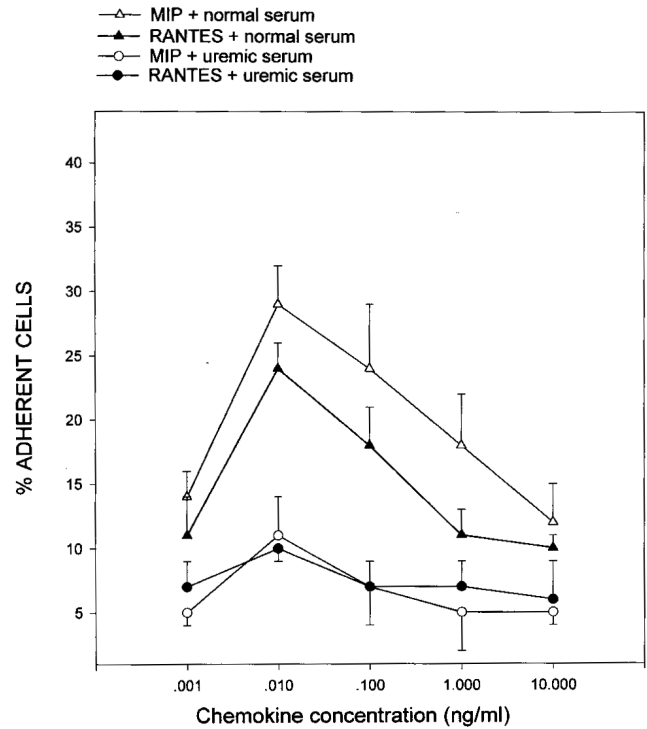
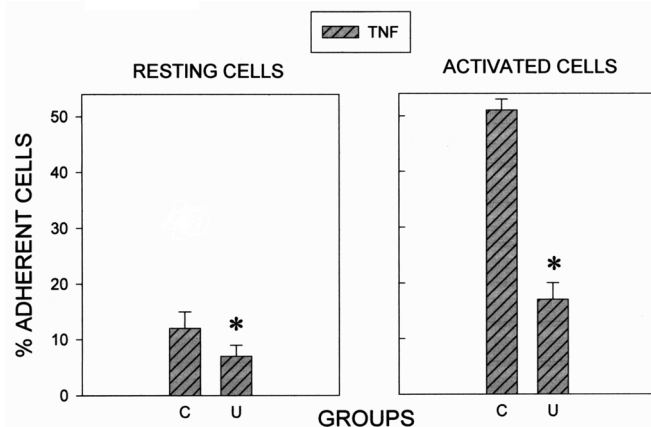**Figure 1.** Adhesion of normal T cells to ECM in response to soluble chemokines.

Figure 2. Adhesion of normal resting and activated T cells to ECM in the presence of normal (C) or uremic (U) serum in response to bound MIP-1b and RANTES. *$P < 0.01$ (resting cells), *$P < 0.02$ (activated cells) for both chemokines.

and 10% vs. 29 and 24% for MIP-1β *and* RANTES, respectively, $P < 0.001$).

- *Effect of uremic serum on induction of adhesion of resting CD4+T cells by ECM-bound chemokines* [Figure 2]. Both MIP-1β and RANTES, bound to intact ECM, retained their adhesion enhancing effect on resting T cells in a non-uremic milieu (39±2 and 37±5% vs. 5±1%, respectively, $P < 0.01$). Uremic serum reduced adhesion of resting cells to ECM to 13±1 and 12±3% for anchored MIP-1β and RANTES, respectively, $P < 0.01$).

- *Effect of uremic serum on induction of adhesion of activated CD4+T cells by ECM-bound chemo-*

Figure 3. Adhesion of normal resting and activated T cells to ECM in the presence of normal (C) or uremic (U) serum in response to TNF-α. *P<0.05 (resting cells), *P<0.01 (activated cells).
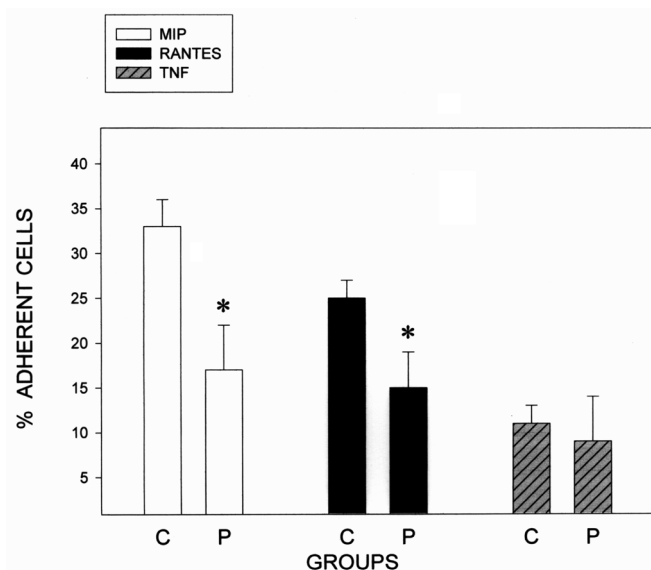
Figure 4. Adhesion of normal (C) or dialysis patients' (P) resting T cells to ECM in response to bound chemokines or TNF-α. *P=0.0001 for MIP-1b and RANTES.

chemokines (Figure 2]. Following PMA stimulation, the pro-adhesive effect of both chemokines on activated T cells was not significant (47±2 and 45±5% for MIP-1β and RANTES, respectively, compared to 41±4% for PMA alone). It was, however, significantly reduced following incubation with uremic serum: 20±2 and 18±2% for MIP-1β and RANTES, respectively, *P<0.02*.

- *Uremic sera effect on TNF-α induced enhancement of resting and activated T cell adhesion to ECM* [Figure 3]. Resting T cells in normal serum demonstrated low binding activity to ECM, even in the presence of immobilized TNF-α (12±3%). However, pre-treatment with PMA enhanced the level of TNF-α-induced binding of T cells to ECM (from 12±3 to 51±2%, *P<0.01*). Upon incubation of T cells with

uremic serum the adhesion ability was reduced, preserving the same pattern of response: 7±2 and 17±3 for resting and activated cells, respectively, *P<0.01* compared to normal cells in a non-uremic environment.

Evaluation of dialysis patients' CD4+T cells

- *Adhesion of resting CD4+T cells (from dialysis patients vs. normal) to ECM in the presence of bound chemokines or TNF-α* [Figure 4]. Percent adherence of dialysis patients' cells to ECM following stimulation with MIP-1β and RANTES was significantly reduced compared to control T cells (17±5 and 15±4% vs. 33±2 and 25±2%, respectively, *P=0.0001*). Adherence to ECM in response to TNF-α was similar among normal and dialysis patients' T cells. There was no difference in cell adherence to ECM between hemodialysis and CAPD patients (data not shown).

Discussion

While chemokines are primarily responsible for chemotaxis, they are also involved in other biological activities. These include degranulation, cytotoxicity, enzyme release [20], cell activation [21], and adhesion to endothelial cells and ECM [14,15]. Cell-matrix adhesion is an essential requirement for a functionally intact immune response [12]. As previously shown, uremic serum inhibits the adhesion capacity of normal CD4+T cells to ECM following PMA stimulation in a dose-dependent manner [17]. Moreover, uremic cells have a reduced adhesive ability, unmodified by incubation in a non-uremic milieu [18].

The soluble chemokines MIP-1β and RANTES increased binding of normal resting CD4+T to ECM in concentrations ranging from 0.001 to 10 ng/ml. Optimal adhesive capacity was observed at 0.01–0.1 ng/ml. Higher concentrations (1–10 ng/ml) did not promote further T cell adhesion. These differing effects of chemokines at various concentrations may allow fine-tuning of chemokine activity, as suggested by previously published data [14,15,21]. Incubation with uremic serum decreased the chemokine-induced adherence of T cells to ECM, implicating the presence of circulating inhibitory substances in uremic serum [6,7,17].

While anchored on ECM, both MIP-1β and RANTES induced approximately 40% binding of resting CD4+T cells to the complexed matrix. These results corroborate other studies which found that cytokines and chemokines, usually thought to act as soluble mediators, can also operate in immobilized forms on ECM glycoproteins [13,15]. In agreement with the literature, PMA-stimulated CD4+T cells did not show any further adhesion on exposure to chemokines [21,22]. In the presence of uremic serum, the adhesive effect of bound chemokines on resting and activated T cells was reduced by almost 75%. The resultant defective adhesion may thereby diminish

the ability of chemokines to act as haptotactic mediators for immune cells at extravascular sites.

In contrast to chemokines, TNF-α-induced T cell adhesion to ECM above the basal level required pre-activation of the cells with PMA. Low doses of bound TNF-α have been reported to enhance PMA-induced integrin-mediated adhesion of CD4+T cells to ECM glycoproteins, fibronectin and laminin [13]. Uremic serum blunted the attachment of normal resting and activated CD4+T cells to ECM following stimulation by TNF-α. T cells of dialysis patients demonstrated a significantly reduced binding to ECM upon stimulation with both chemokines. This was in contradistinction to TNF-α in which the percentage adherence was similar to that found in normal cells.

TNF-α exerts its pro-adhesive effects on T cells via two specific membrane receptors. Hershkoviz et al. [13] have shown that addition of TNF-α binding proteins inhibited the adhesion-enhancing effect of TNF. These soluble truncated portions of the extracellular domains of TNF-αR retain the full ligand-binding activities of the intact receptor and may thus explain the lack of a stimulatory effect of TNF on dialysis patients' T cells [13]. In fact, increased levels of TNF-α and sTNF-αR have been found in uremic plasma [23–25]. Analogous to this is our observation of a diminished TNF-α primed adhesion capacity of normal T cells in the presence of uremic serum, reversed on incubation of patients' T cells in a non-uremic milieu.

The present findings suggest that the previously reported abnormal adhesion properties of T cells to ECM in end-stage renal disease may be related to a decreased stimulatory response to MIP-1β, RANTES or TNF-α, the latter found only in T cells incubated in a uremic environment. The resultant defective adhesion may play a role in the cellular immune dysfunction of uremia.

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