Inhibition of Calcitriol-Induced Monocyte CD14 Expression by Uremic Toxins: Role of Purines

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Abstract. End-stage renal disease is associated with a defect in immunologic functions. Previous studies have demonstrated that uremic ultrafiltrate (UUF) contains factors that suppress calcitriol synthesis and its biological actions. In the present study, the effect of UUF on basal and calcitriol-induced membrane bound CD14 expression of monocytes activated by phorbol 12-myristate 13-acetate was evaluated. CD14 acts as a receptor for the complexes of lipopolysaccharide and lipopolysaccharide-binding protein. Monocytes isolated from normal donors were used for the assay of monocyte CD14 expression. A calcitriol induced rise in monocyte CD14 expression (1966 ± 423 to 2421 ± 436 fluorescence intensity) was found. However, UUF not only suppressed basal CD14 expression of monocytes (from 1966 ± 423 to 1240 ± 203, P < 0.05) but also significantly blunted calcitriol-induced CD14 expression (from 2421 ± 436 to 1744 ± 229, P < 0.05). HPLC fractionated UUF collected from 8 to 16 min (fraction 1, F1) and from 25 to 40 min (fraction 3, F3) also significantly suppressed the expression of CD14. Because ureine derivatives coeluted within F1, their effect on monocyte CD14 expression was also tested. Uric acid, xanthine, and hypoxanthine was found to suppress basal as well as calcitriol-induced CD14 expression of monocytes in a dose-dependent manner. In conclusion, UUF contains factors that impair calcitriol activated function of monocytes.

Although calcitriol (1,25 dihydroxyvitamin D3) is primarily known for its role in mineral metabolism (1), it also plays an important role in immunoregulation (2–4). For instance, calcitriol regulates cytotoxic cells and biosynthesis of immunoglobulins, interleukins, γ-interferon, granulocyte/macrophage colony stimulating factor, tumor necrosis factor, HLA-DR, as well as the differentiation of malignant cells and monocytes (5,6). In addition, calcitriol activates fusion of alveolar macrophages (7) and release of hydrogen peroxide by macrophages (4,8,9).

Several studies have demonstrated that calcitriol has a positive regulatory effect on CD14 expression by leukemic cell lines (10,11). CD14 is a 55-kD glycoprotein expressed in a tissue-specific manner on the surface of mature monocytes and macrophages. This glycoprotein is attached to the cell membrane through a glycosylphosphatidylinositol anchor (12). Because it acts as a receptor for the complexes of lipopolysaccharide (LPS) and LPS-binding protein (13), it plays a major role in immune response.

The activity of NK cells and of mononuclear and polymorphonuclear leukocytes as well as the immune response are altered in renal failure (14–16); because supplementation of calcitriol increases NADPH-oxidase activity in peritoneal mononuclear cells (8), it enhances their phagocytic functions (17–19). Recent studies have shown that uremic ultrafiltrate (UUF) affects the biological action of calcitriol (20).

Therefore, we evaluated the effect of UUF on the basal as well as calcitriol-induced CD14 expression by human monocytes. We found that UUF suppressed both basal and calcitriol-induced CD14 expression.

Materials and Methods

Reagents
RPNI 1640 medium with 2 mM l-glutamine, to which 10% fetal bovine serum (Life Technologies, Merelbeke, Belgium), 1 mM sodium pyruvate, and 50 μg/ml gentamycin were added, was used as the standard culture medium (10). Endotoxins in the culture medium were less than 2 ng/ml by chromogenic limulus amoebocyte lysate assay using Coatest Endotoxin (Kabi-Pharmacia, Uppsala, Sweden).

Phorbol 12-myristate 13-acetate (PMA) (Sigma Chemical Co., Bornem, Belgium) was dissolved in dimethyl sulfoxide, and the stock solution (1 mg/ml) was stored at −20°C. PMA stock solution was diluted (1/100) in sodium acetate (13.6 g/L, pH 6.5) before its use.

Dulbecco’s phosphate buffered saline and trypsin/ethylenediaminetetraacetate (0.5 g of trypsin and 0.2 g of ethylenediaminetetraacetate per liter of modified Puck’s saline A) were obtained from GIBCO BRL, Life Technologies. Calcitriol, kindly provided by Roche (Basel, Switzerland), was dissolved in 95% ethanol and stored at −20°C; the final concentration in the medium was 10 nM (10).

Other materials such as antibodies: Simultest Leucogate (CD45/CD14 [Anti-HLE-1/Leu™-M3]), and Simultest Control γ/γ2a, (IgG1, fluorescein isothiocyanate/IgG2a phycoerythrin) were purchased from
Becton Dickinson (Erembodegem, Belgium). Human IgG was obtained from Sigma Chemical.

HPLC water and HPLC methanol were obtained from Acros organics (Geel, Belgium). Ammonia was purchased from BDH Laboratory Supplies (Poole, UK).

Isolation of the Circulating Blood Monocytes

Blood buffy coats pooled from healthy donors were diluted with sodium citrate (3.2%), and the monocytes were isolated by centrifugation on a Ficoll-Paque density gradient (Pharmacia, Uppsala, Sweden). The monocytes (5 × 10⁶/ml) were washed, resuspended in RPMI 1640 with 10% fetal bovine serum, and incubated for 60 min at 4°C on a roller. Thereafter, these cells were incubated at 37°C for 90 min in a humidified incubator (5% CO₂ in air) on plastic Petri dishes that had been pretreated with fibronectin (1 μg/ml) (Sigma Chemical). The viable cells were harvested from the cultured plates with trypsin/ethylenediaminetetraacetate (0.05%/0.02%) treatment for the following experiments (21). Viability of the monocytes exceeded 90% as assessed at the start of the culture and at the moment of sampling by the trypan blue dye exclusion method.

Preparation of UUF, NUF, and Their Fractions

UFN (Na⁺, 135.7 mmol/L; K⁺, 4.91 mmol/L; creatinine, 10.4 mg/dl; urea, 135 mg/dl; uric acid, 6 mg/dl; xanthine, 0.38 mg/dl; hypoxanthine, 0.96 mg/dl) were pooled from nine patients with chronic hemodialysis. Five patients were treated with low-flux polysulfone dialyzers (Rapido BLS 643, Sorin-Beloco, Mirandola, Italy), and the other four patients with high-flux polysulfone dialyzer (Rapido BLS 627). Ultrafiltrate was collected at the beginning of the hemodialysis session and filtered through a 0.2-μm filter (Minisart NML, Sartorius GmbH, Göttingen, Germany). Each 5 ml of UUF was separated by semipreparative, reversed phase HPLC using a 10-μm Risil C18 column (250 × 10 mm), (BioRad Laboratories S.A.-N.V., Nazareth Eke, Belgium) at a flow rate of 3 ml/min, with a linear gradient of 50 mmol/L ammonium formate, pH 4.0, from 100% to 0%, and methanol of from 0% to 100% over 60 min. This approach resulted in a separation of hydrophilic compounds eluting early during the chromatography and hydrophobic compounds eluting late. HPLC peaks were detected by monitoring of ultraviolet absorption at 254 nm (UVICORD SII, Pharmacia, Bromma, Sweden).

Normal plasma ultrafiltrate (NUF) (Na⁺, 136.4 mmol/L; K⁺, 3.6 mmol/L; creatinine, 1.2 mg/dl; urea, 25 mg/dl; uric acid, 2.9 mg/dl; xanthine, 0.05 mg/dl; hypoxanthine, 0.16 mg/dl) was filtered through an Amicon Centrifio ultrafiltration membrane cone (Danvers, MA), and each 5 ml was subjected to the same HPLC treatment as UUF.

Effects of UUF and NUF on the Expression of Monocyte CD14

Several types of HPLC-prepared UUF and NUF were used to test their effect on the monocyte CD14 expression. We used the UUF and day 3 (after 72 h). Monocytes were cultured in the medium alone or in the medium containing NUF, UUF, F1, F2, F3, or F4 with and without 10 nM calcitriol. In addition, we also studied the effects of xanthine (1.1, 1.5, and 3.0 mg/dl), hypoxanthine (0.125, 0.5, and 1.0 mg/dl), and sodium urate (as these compounds coeluted with F1, see below) on the expression of monocyte CD14. However, because of a variable solubility of the sodium urate in the culture medium (influenced by the concentrations of salts in the RPMI culture medium), addition of sodium urate resulted in variable concentrations with a maximal concentration of 8 mg/dl. Therefore, the final concentrations of uric acid were divided into three groups (mean urate concentration of 3.0, 5.0, and 7.0 mg/dl, respectively). The final uric acid concentrations were measured enzymatically (Sigma Diagnostics). After preparation, the media were filtered to ensure stability [Sterivex-GS 0.22-μm filter unit (Millipore, Bedford, MA)].

Analysis of Membrane Bound CD14 expression

The expression of CD14 on monocytes was assessed by direct immunofluorescence according to the manufacturer’s guidelines. Fifty microliters of the monocytes (approximately 10⁶/ml) were incubated at 37°C for 10 min in the presence of 10 μl of PMA solution (10 mg/L). The samples were then incubated for another 15 min with 12 μl of human IgG diluted in phosphate buffered saline/0.2% bovine serum albumin (to block FcR-binding sites) and 10 μl of Simultest Leucogate at 4°C in the dark. Simultest Leucogate contains fluorescein isothiocyanate-conjugated CD45 monoclonal antibodies (Anti-HLe-1) and phycoerythrin conjugated CD14 monoclonal antibodies (Leu-M3). After washing procedures, the cells were submitted to Flow Cytometric analysis, (FACScan-Becton Dickinson, Erembodegem, Belgium). Fluorescence was standardized by microbeads (Cali- brite particles, Becton Dickinson), and amplification and voltage were kept constant throughout the procedures. Analysis was performed on 5000 events (detector threshold FSC-H:200, parameter FSC-H:1:30).

The monocyte population was gated according to forward and right-angled light scatter. Background binding was estimated by isotype-matched negative control antibodies (Simultest Control).

Analysis of Soluble CD14

Soluble CD14 (sCD14) was measured using sCD14 test kit (ILB, Hamburg, Germany). In brief, monocytes were cultured in control medium without and with 10 nM calcitriol in the absence and in the presence of UUF. The monocytes were stimulated with PMA for 10 min at 37°C and incubated for 15 min at 4°C, followed by centrifugation at 500 × g for 5 min. The supernatant was diluted 1:10 in phosphate buffered saline and incubated for 2 h at room temperature in a microtiter plate precoated with an anti-CD14 oligoclonal antibody. A biotinylated anti-CD14 monoclonal antibody directed against another epitope on the CD14 molecule was simultaneously added. After washing, a streptavadin peroxidase conjugate binds to the bound complex, completing the sandwich. Unbound conjugate is removed by
Results

Membrane Bound CD14 expression on Monocytes

Total UUF. No significant differences in CD14 expression were observed at day 0. The effect at day 2 of UUF on CD14 expression on monocytes cultured in standard culture medium with and without calcitriol is presented in Figure 1. Calcitriol alone stimulated the CD14 expression; however, addition of UUF blunted the CD14 expression with and without calcitriol. Similar results were obtained at day 3. NUF did not affect monocyte CD14 expression (data not shown).

Effects of HPLC Fractions F1 and F3 on CD14 Expression. Both F1 and F3 not only significantly reduced the CD14 expression of monocytes cultured in standard culture medium but also blocked the calcitriol-induced monocyte CD14 expression (Figure 2, A and B). However, F2 and F4 did not inhibit the CD14 expression. In addition, the basic capability of calcitriol to enhance CD14 expression (rise versus standard culture medium (CM), 807 ± 100) was depressed, albeit not significantly, in the presence of F1 (499 ± 127).

HPLC Elution Pattern of UUF

To identify the compounds that were eluted from F1 and F3, we first determined an elution profile of UUF by HPLC. The profile indicated that F1 and F3 were hydrophilic and hydrophobic as exhibited by their respective elution on the left and the right side of the chromatogram. To identify the solutes eluting in the different HPLC fractions, a standard solution containing different known uremic solutes was added to the uremic ultrafiltrate or HPLC solvent. Purine derivatives (uric acid, hypoxanthine, and xanthine) coeluted with F1.

Effect of sodium urate, xanthine, and hypoxanthine on CD14 expression. Figure 3 shows that uric acid suppressed the basal CD14 expression at concentrations of 5.0 and 7.0 mg/dl. Furthermore, both concentrations of uric acid also inhibited the calcitriol-induced CD14 expression. There was no decrease in calcitriol-induced CD14 expression by monocytes at a uric acid concentration of 3.0 mg/dl. Analogous to uric acid, both xanthine and hypoxanthine also blocked the basal as well as calcitriol-induced CD14 expression in a dose-responsive manner (Figure 4, A and B). There was no decrease in calcitriol-induced CD14 expression by monocytes at a hypoxanthine concentration of 0.125 mg/dl.

The capability of calcitriol to enhance CD14 expression (rise versus CM, 1167 ± 226) was also depressed in the presence of a uric acid concentration of 7.0 mg/dl (697 ± 230, P < 0.01) and in the presence of hypoxanthine at concentrations of 0.5 and 1.0 mg/dl (rise versus CM, 1482 ± 428 versus 1158 ± 257 and 1179 ± 283, respectively).

Soluble CD14

Calcitriol upregulated the sCD14 level of monocytes (without calcitriol, 78.6 ± 4.1 µg/L versus with calcitriol, 138.3 ± 3.1 µg/L, n = 3). However, the presence of UUF did not influence either the basal (77.4 ± 6.8 µg/L) or the calcitriol-induced sCD14 levels (140.89 ± 11.0 µg/L, n = 3).

Discussion

Compelling evidence indicates that calcitriol plays an important role in immunomodulation (3–5). For instance, calcitriol inhibits cell proliferation and induces differentiation in a number of cancer cell lines (22–24), it alters the production and regulation of gene expression of various cytokines, such as interleukin-1, interleukin-2, interferon-γ, tumor necrosis factor-α (5). Further, calcitriol also acts as a potent immunosuppressive agent similar to cyclosporin (5). Other biological actions of calcitriol involve regulation of the expression of immune cell membrane components (CD11b, CD14, and CD23) (25).

Previous studies have demonstrated that uremic serum suppressed the calcitriol-induced differentiation of human promyelocytic leukemia cells (HL-60) (26). Patients with end-stage renal disease suffer from an immune deficiency (27) and have increased susceptibility of malignancy (28). Production of calcitriol is decreased in renal failure (29); the combination of uremia and calcitriol deficiency could be partly responsible for abnormal immunoregulation.

The present study was undertaken to evaluate the influence of uremic solutes on calcitriol-induced CD14 expression on monocytes. CD14 is a 55-kD glycoprotein that is attached to the membrane by means of a phosphatidylinositol glycan anchor (12) and has been shown to bind complexes formed by LPS and LPS-binding protein (13).

We found that UUF inhibited the basal as well as calcitriol-induced expression of CD14. Furthermore, we have demonstrated that the UUF compounds inhibiting the CD14 expression eluted in F1 and F3 and that purine derivatives (uric acid, xanthine, and hypoxanthine) coeluted with F1. The purine derivatives exerted a similar inhibitory effect on the CD14 expression analogous to the F1 and UUF. It should be cautioned that the uric acid concentrations used in the present study are at the upper limit of the normal range or only slightly above it. However, the highest values in the dose-response
curve correspond to the mean plasma urate concentrations in our dialysis population (30). Because the solubility of sodium urate was low in the culture medium, consequently it has been impossible to assess the effects of higher concentrations of uric acid. The modulatory effect on CD14 expression has been demonstrated to be dose dependent. An inhibition in CD14 expression is present, irrespective of the absence or the presence of calcitriol. This effect could be interpreted as if UUF would have little effect on the ability of calcitriol to induce CD14 expression. Even without the exogenous addition of calcitriol, this compound may, however, play an active role, in view of the observation that monocytes produce calcitriol themselves (31). In addition it is of note that in the presence of fraction 1, uric acid, and hypoxanthine, the capability of calcitriol to enhance CD14 expression is depressed, albeit only significantly for a uric acid concentration of 7.0 mg/dl.

The question could be raised, why the inhibitory effect in the presence of total UUF is only similar, compared with the individual fractions F1 and F3. One would expect a more pronounced inhibition for total UUF, if the two fractions would act in a cumulative way. However, one cannot exclude that such a summation would be incomplete, whereas total UUF, which contains F1 up to F4, might include compounds that enhance CD14 expression as well.

It should be noted that soluble forms of CD14 (sCD14) were detected in the supernatant of calcitriol-treated myeloid cell line HL-60 (32) and in our calcitriol-treated cells. However, UUF did not inhibit calcitriol-induced sCD14. Therefore, shedding of CD14 from the cell surface into the culture medium could not account for the decreased membrane-bound CD14 of the monocytes. Failure to find a change in soluble CD14 could seem strange if rates of synthesis are really different. Our findings are comparable to those of Cosentino et al., who, in a different setting of immune suppression, also observed an inhibition of CD14 expression on human monocytes in the presence of IL-13, without a change in soluble CD14 (33), which suggests that also in this condition shedding is not involved, and soluble CD14 remains unaffected.

Most of the biological action of calcitriol is a receptor mediated process (genomic pathway). There are several plausible explanations for the inhibitory effects of UUF and purine derivatives: (1) Inhibition of 1α-hydroxylase and calcitriol synthesis by UUF and purine derivatives (34–36). Because monocytes possess 1α-hydroxylase, decreased calcitriol synthesis could account for the lower basal CD14 expression of monocytes incubated with UUF and purines. This mechanism cannot account for the suppression of CD14 expression by exogenous calcitriol; however, it might be involved in the experiments in which no calcitriol is added (2) Inhibition of vitamin D receptor (VDR) synthesis by UUF. Recent studies have shown that UUF reduces the concentrations of VDR of the intestine (37,38) and promyelocytic leukemic cells (26). Thus, UUF could reduce the VDR concentration of monocytes, thereby reducing the expression of calcitriol-induced CD14. (3) Inhibition of the VDR binding to the vitamin D responsive element (VDRE) by UUF; CD14 gene contains VDRE (39) and a recent study indicates that UUF inhibits the VDR binding to the VDRE (40). This inhibitory mechanism could reduce the
expression of CD14, because the interaction of VDR and VDRE results in an inhibition of transcription and synthesis of bioactive proteins. It should be noted, however, that purine derivatives have not been shown to interfere with the binding of VDR to the VDRE.

The biological action of calcitriol is also mediated through a nongenomic pathway. UUF could directly suppress the calcitriol-induced CD14 expression. The nongenomic pathway of calcitriol is a rapid process that occurs within minutes (41). Although we have observed a small decrease in CD14 expression 30 min after addition of uric acid in the presence or absence of calcitriol (data not shown), further experiments are needed to corroborate this finding.

In conclusion, UUF contains factors that inhibit basal as well as calcitriol-induced CD14 expression by monocytes. The suppression of CD14 expression may be of relevance to the impairment of macrophage activation in renal failure. We have also identified that purine derivatives that coeluted with F1 of HPLC processed UUF inhibited the CD14 expression similar to the UUF.

References