Effects of uremic ultrafiltrate on the regulation of the parathyroid cell cycle by calcitriol

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Background. Calcitriol (CTR) is used in the treatment of hyperparathyroidism secondary to renal failure because it decreases parathyroid hormone (PTH) synthesis and parathyroid cell proliferation. Previous studies in tissues other than parathyroids have demonstrated that uremic factors affect the action of CTR on the target cells. We questioned whether the uremic milieu interferes with the inhibition of parathyroid cell proliferation by CTR.

Methods. Studies were performed in vitro using freshly excised normal dog parathyroid tissue incubated for 24 hours with and without CTR and in the presence of either total uremic ultrafiltrate (UUF) from uremic patients or high-pressure liquid chromatography (HPLC)-derived fractions (hydrophilic compounds eluting early and hydrophobic compounds eluting late) of this UUF (F1 to F4). Parathyroid cell proliferation was assessed by flow cytometry.

Results. The addition of CTR $10^{-8}$ and $10^{-7}$ mol/L to parathyroid tissue produced an inhibition of the proliferation that was prevented in the presence of UUF. In a medium containing CTR $10^{-8}$ mol/L, the addition of F1, F2 and F3, but not F4, prevented the CTR-induced inhibition of parathyroid cell proliferation. With CTR $10^{-7}$ mol/L, the inhibition of proliferation was observed even in the presence of F1, F2 and also F4, but was prevented by F3. Uric acid (7 mg/dL), indoxyl sulfate (5 mg/dL) and p-cresol (1.4 mg/dL), which coeluted with F1, F2 and F4, respectively, did not interfere with the inhibitory action of CTR $10^{-7}$ mol/L; however, the addition of phenol (0.14 mg/dL), which coeluted with F3, prevented the CTR-induced inhibition of parathyroid cell proliferation.

Conclusions. The presence of uremic toxins prevents the inhibition of parathyroid cell proliferation induced by calcitriol.

Uremic patients develop progressive parathyroid hyperplasia as a consequence of a decrease in the serum concentration of calcium and calcitriol and a rise in serum phosphate levels. All these factors together stimulate parathyroid cell proliferation. At some time point during the development of parathyroid hyperplasia, there is monoclonal transformation of parathyroid cells and these cells exhibit a decreased expression of receptors for vitamin D and calcium. This explains, at least in part, the poor control of parathyroid cell proliferation by vitamin D and calcium in patients with advanced secondary hyperparathyroidism, which is characterized by parathyroid nodular hyperplasia [1]. Calcitriol is a primary inhibitor of parathyroid cell proliferation by acting on the gene expression of the cell cycle regulator c-myc [2]. However, our recent in vitro study showed that the hyperplastic parathyroid tissue from patients with advanced secondary hyperparathyroidism has a poor response to calcitriol, as a high dose of calcitriol was required to inhibit cell proliferation [3]. These findings could be explained by a decreased VDR expression in advanced parathyroid hyperplasia [4].

Several reports have shown that uremic plasma contains substances that interfere with the action of vitamin D; preincubation of intestinal VDR with uremic ultrafiltrate significantly reduced the interaction of the VDR-hormone complex with DNA [5–7]. However, these studies did not evaluate the effect of uremic ultrafiltrate on parathyroid cells.

The presence of uremic substances potentially affects the regulation of parathyroid cell proliferation by calcitriol. Therefore, we considered it important to determine whether there is an independent effect of uremic toxins on the regulation of parathyroid cell proliferation by calcitriol. The experiments were performed in vitro to avoid confounding effects derived from calcitriol administration such as changes in serum calcium and phosphate.

METHODS

The study was performed using freshly excised parathyroid glands from normal euthanized dogs (2 to 8 years...
of age) from the City Animal Control Service. These dogs were individually housed during a period of 20 days awaiting adoption. During this time the dogs were fed a 1.2% calcium and 0.8% phosphorus diet containing 1600 IU per kg of vitamin D. At euthanization, the two superior parathyroid glands were excised and maintained in culture medium at 4°C until the experiments were performed. The parathyroidectomy required less than five minutes.

Preparation of UUF and the HPLC-derived fractions

Uremic fractions were prepared as previously reported [8]. Uremic ultrafiltrate (UUF) was pooled from nine patients with chronic hemodialysis (with high-flux polysulfone dialyzers, RapidO BLS 627, high permeability membranes; Sorin-Belloco, Mirandola, Italy). Ultrafiltrate was collected at the beginning of the hemodialysis session and filtered through a 0.2 µm filter (Minisart NML; Sartorius GmbH, Gottingen, Germany). Each 5 mL of UUF was separated by semipreparative, reversed phase high-performance liquid chromatography (HPLC) using a 10-µm Rsl C18 column (250 × 10 mm; BioRad Laboratories S.A.-N.V., Nazareth Eke, Belgium) at a low flow rate of 3 mL/min, with a linear gradient of 50 mmol/L ammonium formate, pH 4.0, from 100% to 0%, and of methanol from 0% to 100% over 60 minutes. 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).

Total UUF was reconstituted from the HPLC fractions collected after the first eight minutes of the chromatography, omitting the dead volume and the salt-containing fraction. We tested fractions (F) collected from 9 to 16 (F1), 17 to 24 (F2), 25 to 40 (F3), and 41 to 60 (F4) minutes. The eluates were lyophilized and reconstituted in 5 mL of the culture medium.

Incubation conditions

Experiments were performed using parathyroid tissue slices instead of dispersed parathyroid cells, so that tissue architecture was maintained. Parathyroid glands were cut into small pieces of approximately 1 mm³ and placed in individual wells (24 well dishes from Nunclon Delta SI, InterMed, Denmark) with constant shaking at 37°C and placed in an incubator with a humid atmosphere. The incubation medium was buffered (pH 7.4) and contained in mmol/L: NaCl 125, KCl 5.9, MgCl₂ 0.5, NaH₂PO₄ and Na₂HPO₄ 1 (1:2 ratio), Na-pyruvate 1, glutamine 4, glucose 12 and Hepes 25. Insulin 0.1 IU/mL, bovine serum albumin 0.1%, penicillin G 100 IU/mL, and streptomycin 100 µg/mL were added to the medium. CaCl₂ was added to achieve a final target ionized calcium concentration of 1.25 mmol/L as measured with a selective electrode (634 Ca/pH analyzer; Ciba Corning, Essex, UK). Ionized calcium concentration also was measured in each well after the completion of the experiment and no change in the ionized calcium concentration was observed. The parathyroid tissue was cultured for 24 hours in media without UUF (control) or containing total UUF, F1, F2, F3 or F4, with and without calcitriol (CTR) 10⁻⁸ or 10⁻⁷ mol/L. Additional studies were carried out to evaluate the effect of specific uremic toxins at the same concentration contained in the uremic serum: uric acid (final concentration 7 mg/dL), indoxyl sulfate (5 mg/dL), phenol (0.14 mg/dL) and p-cresol (1.4 mg/dL), as these compounds coeluted within F1, F2, F3 and F4, respectively. The experimental procedure was as previously described for the uremic fractions.

Flow cytometry

Cell proliferation was quantified by flow cytometry; this required isolation of cells that were obtained from the small pieces of parathyroid tissue used in each experimental test period. Small pieces of parathyroid tissue were viewed under an inverted microscope (×10) and were gently stripped apart using a sharp Dumont forceps. This was followed by gentle pipetting. To maintain high cell concentrations, these manipulations were performed in a small volume (50 µL) of phosphate-buffered saline (PBS). The cell cycle was analyzed by a method described by Vindelov and Christensen [9]. Dispersed cells were first treated with PBS containing 0.8% Triton X-100 at 37°C, then with DNAase free RNAase, 10 µg/mL for 10 minutes, and finally with propidium iodide, 20 µg/mL for 30 minutes at 37°C in the dark. Cells were immediately acquired by the flow cytometer (FACScan; Becton-Dickinson, San Jose CA, USA). LYSYS II and CELLFIT software with doublet discrimination module (DDM; Becton-Dickinson) were used for data acquisition and analysis, respectively. Cell debris and clumps were excluded from analysis by gating. This method measures the percent of cells in the different phases of the cell cycle; cells in G₀/G₁ phase are diploid cells; cells in phase S show an increase in the synthesis of DNA which precedes cell duplication and cells in G₂/M have doubled the DNA content. The percent of cells in the S phase was used as a marker of cell proliferation.

Reagents

These included PBS (Oxoid, Hampshire, UK) and calcitriol (Abbott, Madrid, Spain). Uric acid, indoxyl sulfate, phenol, p-cresol, sodium lauril sarkosinate, proteinase K, RNAase, Triton X-100, DNAase free RNAase, propidium iodide, Nonidet-P40, trypsin, trypsin inhibitor and spermine were obtained from Sigma Chemical Co. (St. Louis, MO, USA).
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Fig. 1. Effects of total uremic ultrafiltrate (UUF) and the high-performance liquid chromatography (HPLC)-derived fractions (F1 to F4) after 24 hours of incubation on parathyroid cell proliferation. Data (mean ± SE) show the percent of cells in the S phase of the cell cycle (N = 8).

Fig. 2. Effects of total uremic ultrafiltrate (Total UUF) on calcitriol (10^{-8} and 10^{-7} mol/L)-induced inhibition of parathyroid cell proliferation after 24 hours of incubation. Data (mean ± SE) show the percent of cells in the S phase of the cell cycle (N = 8; *P < 0.01 vs. control without calcitriol).

Statistical analysis

Differences between two means were evaluated by the Wilcoxon test and comparisons of three or more means were evaluated by ANOVA followed by the Duncan test. Results are expressed as the mean ± SE. Data are the means of eight independent duplicated experiments.

RESULTS

Effect of uremic ultrafiltrates on parathyroid cell proliferation

Studies were performed in dog parathyroid tissue incubated for 24-hours without CTR and in the presence of total UUF and the HPLC-derived fractions (F1 to F4). As shown in Figure 1, in control medium (without UUF), the percent of cells progressing to the S phase was 8.4 ± 2.0%. Addition of total UUF or each HPLC-derived fraction, F1, F2, F3 and F4 did not affect significantly the rate of cell proliferation as compared with control.

Effect of UUF on CTR-induced inhibition of parathyroid cell proliferation

Total UUF. The addition of CTR 10^{-8} and 10^{-7} mol/L to parathyroid tissue cultured for 24 hours without UUF produced a significant inhibition of parathyroid cell proliferation (Fig. 2).

Relative to control (no CTR added), the proportion of cells in the S phase was reduced to 54%, P < 0.05 and 44%, P < 0.01 by 10^{-8} and 10^{-7} mol/L of CTR, respectively. By contrast, the percent cells in the S phase in the parathyroid tissue incubated simultaneously with total UUF and CTR were significantly different from that incubated with CTR alone, and not different from the control without CTR. Thus, UUF decreased the ability of CTR to inhibit parathyroid cell proliferation.

HPLC-derived fractions. Experiments with HPLC-derived fractions (F1 to F4) added to the 24-hour culture medium with CTR 10^{-8} and 10^{-7} mol/L are shown in Figure 3 A and B, respectively. In a medium containing CTR 10^{-7} mol/L, fractions F1, F2 and F3 prevented the CTR-induced inhibition of parathyroid cell proliferation. However, in parathyroid tissue cultured with fraction F4, the proportion of cells in S phase relative to control (no calcitriol added) was reduced to 56%, which is similar to the value obtained without the addition of any of the UUF products (53%). Thus, fraction F4 did not appear to interfere with the inhibition of parathyroid cell proliferation induced by CTR.

A higher concentration of CTR, 10^{-7} mol/L, was able to produce inhibition of parathyroid cell proliferation in the presence of fractions F1, F2 and also F4. Relative to control (no calcitriol added) the proportion of cells in S phase was reduced to 45% without UUF and 50%, 36%, 58% with fractions F1, F2 and F4, respectively. Even with this high concentration of calcitriol, fraction F3 prevented a significant decrease in the percent of cells in S phase.

Specific toxins. Experiments were performed to evaluate the effect of specific compounds on the inhibition of parathyroid cell cycle by CTR 10^{-7} mol/L, which was the concentration that showed to better discriminate the effect of the different fractions. The addition of uric acid (7 mg/dL), indoxyl sulfate (5 mg/dL) and p-cresol (1.4 mg/dL) to the medium, which coeluted with F1, F2 and F4, respectively, did not interfere the inhibitory action of CTR 10^{-7} mol/L on parathyroid cell proliferation (Fig. 4). As compared to the control (without calcitriol), the percent cells in the S phase was reduced to 61%, P < 0.01 for uric acid, 46%, P < 0.01 for indoxyl sulfate and 62%,
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Fig. 4. Effects of specific uremic toxins on the inhibition of parathyroid cell proliferation by calcitriol (10^{-8} mol/L) after 24 hours of incubation. Uric acid (final concentration of 7 mg/dL), indoxyl sulfate (5 mg/dL), phenol (0.14 mg/dL) and p-cresol (1.4 mg/dL), which coeluted with F1, F2, F3 and F4, respectively, were analyzed. Data (mean ± SE) show the percent of cells in the S phase of the cell cycle (N = 8; *P < 0.01 vs. control without calcitriol).

DISCUSSION

The present work was designed to investigate whether uremic toxins had an effect on the regulation of parathyroid cell proliferation by calcitriol. The results obtained indicate that uremic toxins prevent the normal inhibition of parathyroid cell proliferation by calcitriol.

The action of CTR on target cells is mediated through the interaction of the CTR receptor (VDR) with the vitamin D response elements (VDRE) of the DNA. Chronic renal failure is often associated with an end-organ resistance to the biologically active form of vitamin D, CTR. Uremic plasma contains compounds, termed uremic toxins, which could account for the diminished biological response to CTR in renal failure, even with normal levels of vitamin D. Uremic toxins have been reported to inhibit the DNA-binding capacity of the hormone-receptor complex with the nuclear chromatin [5–7, 10]. This has been attributed to the formation of Schiff bases between reactive aldehydes and lysine residues of the DNA domain of VDR [7], but experiments on point mutagenesis of the lysine residues could not confirm this hypothesis [11]. A reduction in the VDR levels in the target cells also has been involved in the diminished response to CTR in renal failure [4]. Uremic toxins have been shown to reduce the biological action of calcitriol by suppressing receptor synthesis and the calcitriol induced up-regulation in the gut [10] and promielocytic leukemic cells [12]. In addition, uremic toxins also may have an effect, directly or through the impaired binding of the calcitriol-receptor complex to chromatin, on the activity or the synthesis of specific proteins involved in downstream signaling pathways of calcitriol, as has been reported for the activity of the 1-alpha-hydroxylase [13].

Other functions not directly related to calcium homeostasis have been shown to be altered by uremic toxins such as the case of the CTR-induced monocyte CD14
expression [8], the platelet-activating factor (PAF) synthesis by phagocytic leukocytes, which was abolished by UUF in vitro [14] and the activity of erythrocyte Ca\(^{2+}\)-ATPase [15].

The present study evaluates, to our knowledge for the first time, a direct effect of uremic toxins on the parathyroid glands. The experiments were performed in vitro using parathyroid tissue, which permits an evaluation of an independent effect of uremic toxins and calcitriol on the regulation of parathyroid cell cycle. The administration of calcitriol to animals would have resulted in an increase in calcium, which has separate effects on parathyroid cells. We used parathyroid tissue rather than isolated parathyroid cells because our previous study using the same in vitro model showed an in vitro regulation of parathyroid cell cycle by calcitriol [3]. Our present study analyzed the CaR content of the parathyroid tissue slices maintained in vitro for 24 hours by Western blotting, and showed a conserved expression of this receptor in this model in which the architecture of the tissue was maintained. In addition, in that same model we have evidenced that after a 24-hour incubation the changes in extracellular calcium induced the appropriate variation in PTH release (data not shown). The conservation of the expression of CaR has been reported also in long-term parathyroid cultures from human hyperplastic glands that maintained the tissue architecture as tissue clusters [16]. The requirement of maintaining an intact tissue architecture suggests the involvement of cell-cell interactions or paracrine effects on the parathyroid function, as has been demonstrated by confocal microscopy [17].

Our results showed that culture of parathyroid tissue with total UUF or with each of the four HPLC-derived fractions did not directly enhance the parathyroid cell proliferation. Separate experiments were performed to evaluate the ability of calcitriol to decrease the parathyroid cell proliferation in the presence of uremic ultrafiltrate. While calcitriol (10\(^{-8}\) or 10\(^{-7}\) mol/L) reduces the number of parathyroid cells entering the S phase of the cell cycle, the addition of total uremic ultrafiltrate prevented this effect. The maximal concentration of calcitriol used in the present study was 10\(^{-5}\) mol/L; higher concentrations of calcitriol would have been of little physiological relevance.

The effects of the different HPLC-derived UUF fractions on the inhibition of parathyroid cell cycle by calcitriol also were analyzed. Fractions 1 to 3 impeded the normal inhibition of parathyroid cell proliferation induced by a 10\(^{-5}\) mol/L concentration of calcitriol. Interestingly, the addition of F4 (which contains indole –3 acetic acid, p-cresol and CMPF) to the medium did not interfere with the regulation of parathyroid cell cycle by calcitriol. When calcitriol concentration in the medium was increased to 10\(^{-7}\) mol/L, only F3 (which contains phenol) was able to prevent the inhibition of parathyroid cell cycle induced by this high concentration of calcitriol. These findings suggest that the potent inhibitory effects of uremic ultrafiltrates could result either from the additive effects from the different toxins or from the supraphysiological concentrations used for these in vitro tests, which could limit any extrapolation to the in vivo situation.

To identify uremic toxins that directly affect the inhibition of parathyroid cell proliferation by calcitriol, experiments were performed with specific chemically pure compounds that coelute with the different fractions tested. Uric acid, indoxyl sulfate, phenol and p-cresol, which coeluted with F1, F2, F3 and F4, respectively, were assayed at the concentration range observed in vivo in the uremic serum. All of them showed an effect similar to that observed with the corresponding HPLC-derived fraction. Thus, phenol (eluting with F3) was the compound that blocked the inhibitory effect of CTR on the parathyroid cell proliferation. Phenol has been shown to be elevated in the serum of uremic patients undergoing hemodialysis in the concentration range used in our current study [18]. In a different in vitro model, F1 and F3 impaired the CTR-induced monocyte CD14 expression [8]; purine derivatives (uric acid, xanthine and hypoxanthine), which coelute within F1, were found to suppress basal as well as CTR-induced CD14 expression of monocytes. Phenol and p-cresol, with similar elution pattern as fractions F3 and F4, inhibited PAF synthesis by phagocytic leukocytes [14]. As compounds purified from UUF have different chemical properties (ranging from the most hydrophilic to the most hydrophobic HPLC-derived fractions), some specific affinity must exist for different cell types and functions. Accordingly, regarding the calcitriol-regulated functions, Hsu and Patel reported that the inhibitory effect of UUF on the formation of a VDR-RXR-DNA complex is due to a modification on the VDR, not in the RXR [19]; the alteration of other nuclear receptors cannot be excluded, which could involve some specificity to the toxic effect of uremic toxins.

In hyperplastic parathyroid tissue from patients with advanced secondary hyperparathyroidism we have previously observed that 10\(^{-8}\) mol/L of calcitriol was unable to decrease the parathyroid cell proliferation; however, 10\(^{-7}\) mol/L reduced the parathyroid cell proliferation to 60% of controls. By contrast, in normal dog parathyroid tissue a calcitriol concentration ranging from 10\(^{-11}\) to 10\(^{-7}\) mol/L decreased cell proliferation in a concentration dependent manner [3]. In the present study, experiments were performed in normal parathyroid tissue in which an inhibition of cell proliferation by calcitriol is observed; however, the addition of total UUF and the HPLC-derived fractions F1, F2 and F3 (but not F4) fully abolished the inhibitory effect of calcitriol 10\(^{-8}\) mol/L. Furthermore, total UUF and F3 prevented the action of calcitriol even at a concentration of 10\(^{-7}\) mol/L. The impairment of the effect of calcitriol on parathyroid cell
proliferation caused by uremic toxins, particularly phenol, should not be considered as marginal, as this concentration of calcitriol was able to inhibit cell proliferation in the hyperplastic parathyroid glands in vitro [3].

There are important clinical considerations that are derived from the results of the present study. Uremia itself independently of the serum concentration of calcium, phosphate and calcitriol may play a role in the genesis of the hyperplasia secondary to renal failure. Therefore, treatment of uremia itself should improve the response of the parathyroid cells to the available calcitriol concentration that is decreased in renal failure. Fractions 1 and 2 contain essentially soluble compounds that are easily removed by all of the presently available dialysis systems. According to the present data, this removal should be optimized to allow an optimal impact of calcitriol on the parathyroid glands. F3 contains lipophilic compounds for which the available removal methods are less efficient [20]. Therefore, to obtain an optimal effect, we believe that our concept of dialysis should be modified in a way that lipophilic compounds, with a special mention on phenolic compounds, are efficiently removed also.

In conclusion, the present study demonstrates that uremic toxins affect the inhibition of parathyroid cell proliferation induced by calcitriol.

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