Effects of Uremic Serum on 1,25-Dihydroxyvitamin D₃-Induced Differentiation of a Human Promyelocytic Leukemia Cell Line, HL-60

Masaaki INABA, Senji OKUNO, Hidenori KOYAMA, Yoshiki NISHIZAWA,
and Hirotoshi MORII

*Second Department of Internal Medicine, Osaka City University Medical School,
1-5-7, Asahi-machi, Abeno-ku, Osaka 545, Japan*

Summary The mechanism by which resistance to 1,25 dihydroxyvitamin D₃ (1,25-(OH)₂D₃) occurs in patients with chronic renal failure was studied. This agent induces differentiation and 1,25-(OH)₂D₃-24-hydroxylase activity in the mitochondria of the human promyelocytic leukemia cell line, HL-60, via a steroid-hormone receptor mechanism. HL-60 cells were cultured in RPMI 1640 medium supplemented with 10% normal or uremic serum. Treatment of these cells with 10⁻⁸M 1,25-(OH)₂D₃ for 5 days in a medium containing 10% uremic serum from 4 patients with chronic renal failure resulted in a maturation of the cells amounting to 30.3 ± 18.7% (mean ± SD) and 32.5 ± 11.2%, as obtained by NBT reduction assay and NSE assay, respectively. These values were significantly lower than those obtained with 10% serum from 3 normal controls (66.6 ± 12.8%, 58.3 ± 10.9%, *p* < 0.02). The treatment of HL-60 cells with 1,25-(OH)₂D₃ in a mixture of 5% normal plus 5% uremic serum caused cell differentiation to an extent similar to that in 10% uremic serum, which suggests the presence of a substance(s) having 1,25-(OH)₂D₃-inhibitory activity in the uremic serum. Exposure of HL-60 cells to uremic serum significantly impaired their responsiveness to 1,25-(OH)₂D₃ as assessed by the induction of the cell's ability to hydroxylate the C-24 position of 1,25-(OH)₂[³H]D₃. The mechanism by which uremic serum confers an impaired cellular response to 1,25-(OH)₂D₃ seemed to be due, in part, to a decrease in 1,25-(OH)₂D₃ receptor levels. A significant positive correlation was observed between intracellular cAMP levels and 1,25-(OH)₂D₃-induced HL-60 cell maturation. In summary, the mechanism by which uremic serum confers 1,25-(OH)₂D₃ resistance upon HL-60 cells seemed to be due to the presence of 1,25-(OH)₂D₃-inhibitory activity in uremic serum, which may modulate cellular responsiveness to 1,25-(OH)₂D₃ by such mechanisms as reducing 1,25-(OH)₂D₃ receptor levels in the cells, in part through alteration in cAMP metabolism.

Key Words 1,25-dihydroxyvitamin D, vitamin D, HL-60, uremia, hyperparathyroidism

1,25-Dihydroxyvitamin D₃ (1,25-(OH)₂D₃) induces the human promyelocytic leukemia cell line, HL-60, to differentiate into monocytes/macrophages *via* a steroid-hormone receptor mechanism (1-6). Further, 1,25-(OH)₂D₃ induces 1,25-(OH)₂D₃-24-hydroxylase in HL-60 cell mitochondria in a dose-dependent manner (7) and induces up-regulation of the 1,25-(OH)₂D₃ receptor (8). These data clearly indicate that the HL-60 cell system is appropriate for the investigation of the molecular mechanism of 1,25-(OH)₂D₃ action. Many recent reports have indicated the occurrence of resistance to 1,25-(OH)₂D₃ in patients with chronic renal failure (9-11); the mechanism for this resistance has been explained, in part, by a reduction in the number of 1,25-(OH)₂D₃ receptors (12-14). It has also been reported that chronic renal failure is associated with a significant impairment of immune potential, which could be returned to normal by the administration of 1 α -hydroxyvitamin D₃ (15), and that uremic serum contains humoral factors which affect various functions of peripheral blood mononuclear cells *in vitro* (16,17). Furthermore, uremic toxins have been reported to affect the proliferation of HL-60 cells (18). Recent reports have demonstrated the intimate involvement of 1,25-(OH)₂D₃ in the immune system (19,20). The HL-60 cell system is the most intensively investigated model within the cell-mediated immune system for examining the effects of 1,25-(OH)₂D₃. These reports led us to examine the influence of uremic serum on the effects of 1,25-(OH)₂D₃ on HL-60 cells.

MATERIALS AND METHODS

Cells and Cell culture. HL-60 cells, provided by Dr. Negoro (First Department of Internal Medicine, Osaka City University Medical School), were cultured as described previously (3,4). Control sera were drawn from 3 normal healthy males and uremic sera were drawn from 4 males with chronic renal failure. The latter had not received dialysis therapy and had no history of taking medications which might affect Ca metabolism, such as vitamin D₃ derivatives or aluminum-containing phosphate binders. Their renal disorders were all of glomerulonephritic etiology, with no evidence of other metabolic disorders such as diabetes mellitus. The serum parameters, BUN, creatinine, Ca, Pi, and Mg were assayed by an automated technique, and serum PTH levels were assayed by RIA, using antibody raised against the synthetic midportion of PTH (HS-PTH, Yamasa Shoyu Co., Choshi, Japan). Although the normal control and uremic patients were not matched for age, we observed no dependence of 1,25-(OH)₂D₃-induced cell differentiation upon age in normal controls (data not shown). In the experiments to assess differentiation and intracellular cAMP levels, HL-60 cells were inoculated at 2.0×10^5 cells/ml and cultured in medium supplemented with 10% of an individual serum. In other experiments to assess the content of 1,25-(OH)₂D₃ receptors and the capacity of the cells to synthesize 1,24,25-(OH)₃[26,27-³H]D₃, cells were inoculated at 2.0×10^6 cells/ml and cultured in medium containing 10% control or uremic serum. Because these experiments required a large volume of serum, pooled uremic serum was used, whereas control serum was obtained from one normal subject. Cells were counted with a hemacytometer, and cell viability was determined by the trypan blue exclusion method. HL-60 cell differentiation

was assessed by NBT reduction assay (3) and nonspecific esterase (NSE) assay, using a commercially available kit (Sigma No 90, St Louis, MO), as described previously (21). After HL-60 cells were cultured in medium containing 10% normal or uremic serum for the specified times, cell extracts for receptor determination were processed as previously described (4,6). Levels of 1,25-(OH)₂D₃ receptors were determined as previously described (6). After the HL-60 cells were cultured in medium containing 10% normal or uremic serum for the specified times, they were assayed for their ability to hydroxylate 1,25-(OH)₂[26,27-³H]D₃ to form 1,24,25-(OH)₃[26,27-³H]D₃, using the periodate-based method, as previously described (7,21,23). Intracellular cAMP levels were determined with a cAMP-RIA kit (25), kindly provided by Yamasa Shoyu Co., Ltd (Choshi, Japan).

All data were expressed as mean $\pm$ SD. Statistical analysis was done with Student's *t* test. A level of *p* < 0.05 was regarded as statistically significant.

RESULTS

Effects of normal or uremic serum on 1,25-(OH)₂D₃-induced differentiation of HL-60 cells.

HL-60 cells were cultured in RPMI 1640 medium supplemented with 10% individual serum obtained from normal subjects or uremic patients. Profiles and serum parameters of the study participants appear in Table 1. Serum BUN, creatinine, and hsPTH levels were significantly elevated in the uremic patients as compared with the control subjects. The uremic patients also tended to have an increased serum level of phosphate and Mg. Treatment of the HL-60 cells with 10⁻⁸ M 1,25-(OH)₂D₃ led to a time-dependent increase in the rate of cell differentiation (data not shown). After incubation for 5 days with 10⁻⁸ M 1,25-(OH)₂D₃, 66.6 $\pm$ 12.8% of the HL-60 cells reduced NBT; 58.3 $\pm$ 10.9% of them expressed NSE activity when cultured in normal serum (*n*=3). However, only 30.3 $\pm$ 18.7% and 32.5 $\pm$ 11.2% of the cells cultured in uremic serum (*n*=4) acquired the characteristics of monocytes/macrophages, as determined by NBT reduction and NSE assay, respectively (*p* < 0.02; Table 2). Considering the impaired 1,25-(OH)₂D₃-induced maturation in uremic serum, the finding that cell counts after 5 days of treatment were similar in the control- and uremic serum-treated cells indicates that the proliferation of

Table 1. Profiles and serum parameters of subjects enrolled in the study

Subjects	Serum Parameter	BUN* (mg/dl)	Cre* (mg/dl)	Ca (mEq/liter)	Pi (mg/dl)	Mg (mg/dl)	hsPTH* (pg/ml)
Controls							
Male, 28 y.o.		14.0	1.1	4.5	4.1	1.8	284.0
Male, 32 y.o.		16.6	1.1	4.6	4.8	2.1	424.0
Male, 36 y.o.		14.9	1.1	4.6	4.5	1.9	360.0
Uremic Patients							
Male, 68 y.o.		55.4	3.9	5.2	5.8	2.3	1747.0
Male, 77 y.o.		73.7	4.5	4.5	6.0	1.7	1829.0
Male, 65 y.o.		53.4	6.9	4.2	5.2	2.0	3200.0
Male, 56 y.o.		59.1	4.3	4.0	4.5	2.1	4120.0

*Significant difference (*p* < 0.05) between control and uremic patients (Student's *t* test)

HL-60 cells was suppressed by treatment with uremic serum, as reported previously (18).

Mixing study

To determine whether 1,25-(OH)₂D₃-inhibitory activity might be present or whether a substance(s) essential for the 1,25-(OH)₂D₃ effect might be deficient in uremic serum, the differentiation-inducing effect of 1,25-(OH)₂D₃ was assessed in cells treated with a combination of 5% normal and 5% uremic serum. As shown in Fig. 1, the proportion of mature cells cultured in mixed serum as assessed by NBT reduction and NSE assay was $54.0 \pm 3.9\%$ and $50.2 \pm 5.8\%$ ($n=3$), respectively, similar to the values obtained with 10% uremic serum ($52.0 \pm 5.8\%$, $49.9 \pm 2.5\%$, $n=3$), but significantly less than those with

Table 2. Effects of uremic serum, compared to normal serum, on 1,25-(OH)₂D₃-induced cell differentiation.

Treatment	Assessment	Proliferation ($\times 10^6$ cells/ml)	Differentiation	
			NBT reduction assay	NSE assay
Normal serum ($n=3$)		1.23 ± 0.17	$66.6 \pm 12.8\%$	$58.3 \pm 10.9\%$
Uremic serum ($n=4$)		1.27 ± 0.23	$30.3 \pm 18.7\%$	$32.5 \pm 11.2\%$
Significance		$p=0.410$	$p<0.02$	$p<0.02$

Fig. 1. Effects of uremic serum on 1,25-(OH)₂D₃-induced differentiation of HL-60 cells. HL-60 cells were cultured with 10^{-8} M 1,25-(OH)₂D₃ for 5 days in RPMI 1640 medium containing 10% control serum, uremic serum, or a combination of 5% control plus 5% uremic serum. Differentiation was assessed by NBT reduction assay (▨) and NSE assay (□), as described in METHODS.

normal serum ($82.3 \pm 4.7\%$, $74.6 \pm 2.0\%$, $n=3$, $p < 0.005$). These results indicate that the occurrence of 1,25-(OH)₂D₃-resistance in cells treated with uremic serum was a cumulative phenomenon, suggesting that a substance(s) having 1,25-(OH)₂D₃-inhibitory activity was present in the uremic serum.

Effect of normal and uremic serum on 1,25-(OH)₂D₃ induction of cell activity to hydroxylate C-24 of 1,25-(OH)₂[26,27-³H] D₃

We recently demonstrated that 1,25-(OH)₂D₃ specifically induces 1,25-(OH)₂D₃-24-hydroxylase activity in HL-60 cell mitochondria in a dose-dependent manner (7). The effects of uremic pooled serum on the induction by 1,25-(OH)₂D₃ of 24-hydroxylation activity of 1,25-(OH)₂[26,27-³H] D₃ in HL-60 cells were compared with the effects of normal serum. After the cells had been treated with either kind of serum for 1 or 3 days, the induction of 24-hydroxylation activity by 1,25-(OH)₂D₃ was found to be significantly less in cells treated with uremic serum than in those treated with normal serum (Fig. 2), supporting the hypothesis that uremic serum may contain 1,25-(OH)₂D₃-inhibitory activity.

Fig. 2. Effects of uremic serum on induction of the ability of HL-60 cells to hydroxylate C-24 of 1,25-(OH)₂[26,27-³H] D₃. HL-60 cells were cultured in RPMI 1640 medium containing 10% control (□) or uremic pooled (■) serum for 1 day (left panel) and 3 days (right panel). After the cells were treated with the indicated concentrations of 1,25-(OH)₂D₃ for 24 h, they were assayed for ability to form 1,24,25-(OH)₃[26,27-³H] D₃, using the periodate-based assay, as described in Methods.

Effects of normal and uremic serum on 1,25-(OH)₂D₃ receptor content of HL-60 cells

The 1,25-(OH)₂D₃-resistance observed in uremic patients has been explained, in part, by a reduction in 1,25-(OH)₂D₃ receptor levels in target tissues (12–14). Therefore, the 1,25-(OH)₂D₃ receptor content was determined in HL-60 cells after exposure to either-type of serum. The K_d for specific 1,25-(OH)₂D₃ binding calculated from the slope of the regression line in HL-60 cell extract was 5.60×10^{-11} M, indicating the presence of specific 1,25-(OH)₂D₃ receptors in the cells. Treatment of the cells with uremic serum for 1 day led to a significant reduction in the 1,25-(OH)₂D₃ receptor levels, whereas 3 days of treatment did not (Fig. 3).

Effects of uremic serum on the intracellular cAMP content of HL-60 cells

We have observed previously that addition of dbcAMP to the culture medium of HL-60 cells enhanced the 1,25-(OH)₂D₃-mediated induction of cell differentiation and 24-hydroxylation activity in these cells; the mechanism of enhancement being explained, in part, by an increase in 1,25-(OH)₂D₃ receptor content (Inaba, M. *et al.* unpublished data). Together with the findings that uremic serum confers 1,25-(OH)₂D₃-resistance upon HL-60 cells in the induction of cell differentiation and in the activity to hydroxylate C-24 of 1,25-(OH)₂[26,27-³H]D₃, partly by decreasing the 1,25-(OH)₂D₃ receptor content, these data raise the possibility that a possible decrease in intracellular cAMP level might contribute to the 1,25-(OH)₂D₃-resistance observed in the uremic serum-treated cells. Intracellular cAMP levels were determined after cells were cultured for 2 days in 10% serum from 3 control subjects, or in 10% serum from 4 uremic patients. An incubation time of 2 days was selected because after 2 days' exposure to 1,25-(OH)₂D₃, the cells so

Fig. 3. Effects of uremic serum on 1,25-(OH)₂D₃ receptor content in HL-60 cells. HL-60 cells were cultured in RPMI 1640 medium containing 10% control (□) or uremic (▨) serum for 1 or 3 days. 1,25-(OH)₂D₃ receptor content was assessed by calculating the specific binding; this was done by subtracting the non-specific from the total binding, as described in Methods.

Fig. 4. Positive correlation between intracellular cAMP content and 1,25-(OH)₂D₃-mediated differentiation-inducing effect, assessed by NBT reduction and NSE assays. Intracellular cAMP levels were determined by RIA after 2 days of treatment with either control or uremic serum. The differentiation-inducing effect of 1,25-(OH)₂D₃ was assessed, after 5 days of treatment with 10^{-8} M 1,25-(OH)₂D₃ by the NBT reduction assay (left panel) and the NSE assay (right panel). Reproduced from Inaba *et al.*, with permission of the publisher.

treated progress from the commitment step to the phenotype-expression step (26). Intracellular cAMP levels in the uremic serum-treated cells ($n=4$) were 9.85 ± 1.16 pmol/ 10^7 cells, as compared with 13.7 ± 3.20 pmol/ 10^7 cells in the normal serum-treated cells ($n=3$, $p < 0.05$). As shown in Fig. 4, cAMP levels in the cells treated with control serum for 2 days and levels in cells similarly treated with uremic serum were significantly correlated with the cells' responsiveness to 1,25-(OH)₂D₃ in the induction of cell differentiation, as assessed by the NBT reduction assay ($p < 0.05$); a similar tendency was observed with the NSE assay ($p = 0.082$).

DISCUSSION

In this study, we demonstrated that the treatment of HL-60 cells with uremic serum as compared to normal serum, significantly attenuated the effects of 1,25-(OH)₂D₃ on the induction of cell differentiation and the ability of cells to hydroxylate C-24 of 1,25-(OH)₂ [26,27-³H] D₃. The results obtained from a mixing study strongly suggested the presence of a substance(s) which inhibited the action of 1,25-(OH)₂D₃ on these cells. Also, a significant suppression of cell proliferation was observed with uremic serum treatment, as described previously (18). However, as HL-60 cell differentiation is not coupled to its proliferation, as we (3,21) and others (27,28) have previously demonstrated, the proliferation-suppressive effect of uremic serum could be neglected in assessing the effects of 1,25-(OH)₂D₃ on cell differentiation. Although Ca and Mg levels in the culture medium

are known to affect 1,25-(OH)₂D₃-induced HL-60 cells differentiation (29,30), we did not detect a significant difference between those levels in the uremics and the controls. Aluminum is known to cause 1,25-(OH)₂D₃ resistance at a post-receptor step, with a compensatory rise in 1,25-(OH)₂D₃ receptor levels in target tissues (31). However, the uremic patients enrolled in this study had received no medication containing aluminum, nor were they being dialyzed with water of high aluminum content. Furthermore, even if the difference in serum 1,25-(OH)₂D₃ levels between control and uremic patients had been of statistical significance, the 1,25-(OH)₂D₃ levels in culture medium containing 10% human serum should have been of the order of 10⁻¹¹ M. Because we evaluated the cellular responsiveness to 1,25-(OH)₂D₃ at concentrations as high as 10⁻⁹–10⁻⁷ M, the possibility could be excluded that the difference in endogenous 1,25-(OH)₂D₃ levels between control and uremic serum contributed to the difference in cell maturation. The finding that the 1,25-(OH)₂D₃ receptor content of HL-60 cells was decreased significantly by exposure to uremic serum for 1 day supports the recent hypothesis that the 1,25-(OH)₂D₃ resistance seen in uremia may be caused by down-regulation of the 1,25-(OH)₂D₃ receptors. However, no significant decrease in 1,25-(OH)₂D₃ receptor content was observed in cells treated with uremic serum for 3 days, whereas those cells responded to 1,25-(OH)₂D₃ to a significantly lesser extent in the induction of 24-hydroxylation activity vs. the cells treated with normal serum. We recently observed that the 1,25-(OH)₂D₃-induced up-regulation of intestinal 1,25-(OH)₂D₃ receptors was significantly impaired in 5/6 nephrectomized uremic rats vs. sham-operated rats (Koyama H. *et al.*, unpublished data). Because we used an incubation time of 24 h for the 24-hydroxylase induction experiment, the 1,25-(OH)₂D₃-induced up-regulation of 1,25-(OH)₂D₃ receptors during the incubation period may have been impaired in the cells treated with uremic serum. However, these data raise the possibility that the occurrence of 1,25-(OH)₂D₃ resistance might be the result not solely of a decrease in 1,25-(OH)₂D₃ receptor content, but also of some other unknown mechanisms. We have recently observed that dbcAMP enhanced the effects of 1,25-(OH)₂D₃, both on the induction of HL-60 cell differentiation along the monocyte/macrophage pathway and on the induction of 1,25-(OH)₂D₃-24-hydroxylation activity, and that dbcAMP caused a heterologous up-regulation of 1,25-(OH)₂D₃ receptors, indicating that an increase of intracellular cAMP levels enhances the effects of 1,25-(OH)₂D₃, at least in part, by increasing 1,25-(OH)₂D₃ receptor levels (Inaba, M. *et al.* unpublished data). Thus, the phenomena observed in the cells treated with uremic serum were in sharp contrast to those observed in dbcAMP-treated cells. A previous report has demonstrated that the content of ATP, ADP, and AMP was decreased in the tissues of uremic rats (32). Taken together with the data that cAMP levels in uremic serum-treated cells were significantly lower than those in control serum-treated cells, it is possible that a decrease in cAMP content might be responsible for the occurrence of 1,25-(OH)₂D₃ resistance in the uremic state. As supportive of this hypothesis, significant positive correlation observed between intracellular cAMP levels and the differentiation-inducing effects of 1,25-(OH)₂D₃ (Fig. 4).

Patients with chronic renal failure, in whom 1,25-(OH)₂D₃ resistance has been reported, showed parathyroid gland hyperplasia. Generally, intracellular cAMP levels

were negatively correlated with the rate of cell proliferation (33). Therefore, in further examination of the hypothesis that cAMP plays a modulatory role in the effects of 1,25-(OH)₂D₃ on various 1,25-(OH)₂D₃ target tissues, it would be interesting to evaluate the influence of cAMP on the potency of the 1,25-(OH)₂D₃-induced effect on parathyroid cells, which also revealed 1,25-(OH)₂D₃-resistance in the uremic state.

In summary, uremic serum contains a substance(s) which confers 1,25-(OH)₂D₃-resistance upon HL-60 cells in the induction of cell differentiation and 24-hydroxylation activity. The mechanism by which 1,25-(OH)₂D₃-inhibitory activity conferred 1,25-(OH)₂D₃-resistance upon the HL-60 cells seemed to be due, in part, to a reduction of 1,25-(OH)₂D₃ receptor numbers in those cells, which may be mediated by a decrease in intracellular cAMP levels.

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