p21\textsuperscript{WAF1} and TGF-\(\alpha\) mediate parathyroid growth arrest by vitamin D and high calcium

MARIO COZZOLINO, YAN LU, JANE FINCH, EDUARDO SLATOPOLSKY, and ADRIANA S. DUSSO

Renal Division, Department of Internal Medicine, Washington University School of Medicine, St. Louis, Missouri, USA, and Renal Division, Ospedale San Paolo, Milano, Italy

\textbf{Background.} High dietary phosphorus (P) worsens uremia-induced parathyroid (PT) hyperplasia through increases in the growth promoter transforming growth factor-alpha (TGF-\(\alpha\)). In contrast, P restriction prevents PT hyperplasia by inducing the cell cycle inhibitor p21. Since 1,25(OH)\textsubscript{2}D\textsubscript{3}-antiproliferative action in various cell types involves increases in p21, we studied whether induction of p21 by 1,25(OH)\textsubscript{2}D\textsubscript{3} or the vitamin D analog, 19-Nor-1,25(OH)\textsubscript{2}D\textsubscript{2}, could counteract the PT hyperplasia induced by high dietary P in early uremia.

\textbf{Methods.} Normal (N) and uremic (U; 5/6 nephrectomized) female Sprague-Dawley rats were fed high P (HP), low P (LP) or high Ca (HCa) diets and administered intraperitoneally (IP) either vehicle or vitamin D metabolites for seven days, as follows: N-HP; U-HP + vehicle; U-HP + 1,25(OH)\textsubscript{2}D\textsubscript{3} (4 ng/day); U-HP + 19-Nor-1,25(OH)\textsubscript{2}D\textsubscript{2} (30 ng/day); U-LP; U-HCa. Serum PTH and PT gland weight assessed secondary hyperparathyroidism. Immunohistochemical quantitation of two markers of mitotic activity, Ki67 and PCNA measured PT hyperplasia. Immunohistochemical expression of PT p21 and TGF-\(\alpha\) addressed potential mechanisms mediating the opposing effects of high and low dietary P on PT cell growth demonstrated that high dietary P worsens uremia-induced PT hyperplasia by increasing PT content of the cyclin-dependent kinase inhibitor, p21 [6].

\textbf{Results.} 1,25(OH)\textsubscript{2}D\textsubscript{3} and 19-Nor-1,25(OH)\textsubscript{2}D\textsubscript{2} were effective in suppressing both PTH secretion and PT hyperplasia induced by uremia and high dietary P independent of increases in ionized Ca. Both vitamin D compounds enhanced PT p21 expression and prevented high P-induced increases in PT TGF-\(\alpha\) content. Induction of PT p21 and reduction of TGF-\(\alpha\) content also occurred when uremia-induced PT hyperplasia was suppressed by high dietary Ca.

\textbf{Conclusions.} In early uremia, vitamin D suppression of high P-induced PT hyperplasia and high dietary Ca arrest of PT growth involve induction of PT p21 and prevention of increases in TGF-\(\alpha\).

The control of parathyroid (PT) hyperplasia is crucial in preventing secondary hyperparathyroidism in uremic patients [1]. The enlargement of the PT glands results in enhanced synthesis and secretion of parathyroid hormone (PTH), which in turn increases bone resorption, a feature of renal osteodystrophy [2]. Under physiological conditions, PT cells remain quiescent [1–3]. However, they retain their potential to proliferate in response to certain stimuli, such as uremia, low calcium (Ca) intake, vitamin D deficiency, and high dietary phosphorus (P) [4, 5]. Although the mechanisms by which these factors control PTH biosynthesis and secretion are well known, the mechanisms responsible for their effects on PT cell proliferation are poorly understood.

The most efficient way to study the regulation of PT tissue growth, synchronized PT cell cultures, is hindered by the lack of an appropriate PT cell line. Therefore, the experimental approach for analyzing the rates of PT cell proliferation and apoptosis is limited to the in vivo uremic rat model. Studies in our laboratory assessing potential mechanisms mediating the opposing effects of high and low dietary P on PT cell growth demonstrated that high dietary P worsens uremia-induced PT hyperplasia by increasing PT expression of the growth promoter transforming growth factor alpha (TGF-\(\alpha\)) [6]. In contrast, dietary P restriction induces PT growth arrest by increasing PT content of the cyclin-dependent kinase inhibitor, p21 [6].

These studies addressed potential mechanisms for the regulation of PT cell growth by 1,25(OH)\textsubscript{2}D\textsubscript{3} and its analog 19-Nor-1,25(OH)\textsubscript{2}D\textsubscript{2}. Several laboratories demonstrated a role for 1,25(OH)\textsubscript{2}D\textsubscript{3} in the control of parathyroid cell proliferation in vitro [7], and in vivo in uremia-induced PT hyperplasia in 5/6 nephrectomized rats [8]. Based on the reported antiproliferative properties of 1,25-dihydroxyvitamin-D\textsubscript{3} in PT cells, we first studied the efficacy of vitamin D treatment [1,25(OH)\textsubscript{2}D\textsubscript{3} and its analog 19-Nor-1,25(OH)\textsubscript{2}D\textsubscript{2}] to counteract the PT hyperplasia induced by high dietary P in early uremia. In cells of the monocyte-macrophage lineage [9] as well as in other cell types [10–12], vitamin D suppresses cell growth through the induction of p21. Therefore, to identify potential mechanisms mediating the inhibition of PT cell proliferation by vitamin D treatment, we first examined
the contribution of increased PT-p21 expression. Since TGF-α content directly correlates with the degree of PT hyperplasia induced by high dietary P, we also examined the effects of vitamin D treatment on PT TGF-α expression. Finally, we compared the effects of vitamin D treatment on PT p21 and TGF-α content with those induced by high dietary Ca, a well-known promoter of PT growth arrest in uremia.

Our results show that vitamin D treatment may prevent high P-induced PT hyperplasia by inducing p21 and preventing increases in TGF-α expression. The suppression of uremia-induced PT hyperplasia by high dietary Ca also involves changes in PT p21 and TGF-α content similar to those induced by vitamin D therapy, thus supporting these two molecules as important regulators of PT cell growth.

METHODS

Normal and uremic (5/6 nephrectomized) female Sprague-Dawley rats, 5 to 6 weeks old, weighing 225 to 250 grams were studied. For 5/6 nephrectomy, several branches of the left renal artery were ligated and the right kidney excised. Rats underwent one of the following experimental conditions: (a) normal rats fed a high P diet (0.9% P; 0.6% Ca) (N-HP); (b) uremic rats fed the same high P diet (U-HP); (c) uremic rats fed the high P diet and treated with 4 ng/day of 1,25(OH)2D3 (U-HP + 1.25D); (d) uremic rats fed the high P diet and treated with 30 ng/day of 19-Nor-1,25(OH)2D2 (U-HP + 19Nor); (e) uremic rats fed a low P diet (0.2% P; 0.5% Ca) (U-LP); and (f) uremic rats fed a high Ca diet (1.25% P; 2.0% Ca) (U-HCa). Diets were purchased from Diets, Inc. (Bethlehem, PA, USA). Both 1,25(OH)2D3 and 19-Nor-1,25(OH)2D2 were administered intraperitoneally (IP) in 100 μL of propylene glycol. All other experimental groups received 100 μL of propylene glycol daily as vehicle. The doses of 1,25-dihydroxyvitamin D3 (4 ng daily) and 19-Nor-1,25-dihydroxyvitamin D2 (30 ng daily) were chosen from pilot studies designed to control PT hyperplasia while avoiding hypercalcemia.

After a seven-day treatment, rats were sacrificed. Blood was drawn for analytical determinations. PT glands were surgically removed and weighed on a CAHN-31 microbalance (Cahn Instruments, Inc., Cerritos, CA, USA). For immunohistochemistry, PT glands were kept in 10% formaldehyde overnight and then transferred to 70% ethanol in water before mounting. All experimental protocols were approved by the Animal Study Committee at Washington University School of Medicine.

Analytical determinations

Ionized calcium (ICA) was measured by an ICA-specific electrode (Model ICA-1; Radiometer, Copenhagen, Denmark). Plasma P and creatinine were determined using an autoanalyzer (COBAS-MIRA Plus, Branchburg, NJ, USA). Serum total Ca was measured by atomic absorption spectrophotometry using a Perkin-Elmer 1100B spectrophotometer (Perkin-Elmer, Norwalk, CT, USA). Intact PTH levels were measured by an immunoradiometric assay specific for intact rat PTH (Immunotopics, San Clemente, CA, USA).

Immunohistochemistry

Immunohistochemical staining for Ki-67 related antigen (Ki67), proliferating cell nuclear antigen (PCNA), p21, and TGF-α was performed on sections of formalin-fixed, paraffin embedded PT glands following protocols described in previous studies for each specific protein [6]. For Ki67 immunostaining, a mouse monoclonal anti-Ki67 antibody (MIB-5, Cat. No.2093; Immunotech, Marseille, France) was used as the primary antibody. Specificity of the primary antibodies was tested by immunohistochemical staining of rat PT tissue replacing the primary antibody with mouse IgG1. For TGF-α immunostaining, PT tissue was pretreated with 0.05% saponin for 30 minutes at room temperature. Tissue was then blocked with 10% preimmune goat serum and incubated with primary antibody (4 μg/mL for Ki67, 1.13 μg/mL for PCNA, 5 μg/mL for p21, and 10 μg/mL for TGF-α) for 12 hours at room temperature. Twenty-four consecutive sections of tissue were cut for each PT gland. Immunohistochemical staining of Ki67, PCNA, p21, and TGF-α proteins were quantitated. Ki67 and PCNA-positive nuclei were counted in ten high-power fields (HPF) and expressed as the number of positive-staining nuclei per area, which included sections of the whole PT glands. Positive staining for p21 was graded as: (–) negative, (+) <25%, (++) 25 to 50%, and (+++) >50%. Grades ++ and +++ were taken as positive staining. Immunohistochemical staining was evaluated independently by three different individuals who were blinded to the origin of the samples. Ten different slides were analyzed for each experimental condition.

Immunohistochemical staining of Ki67, PCNA, and TGF-α proteins was quantitated using a Nikon Diaphot-TMD microscope coupled to a camera and an image analysis system. Images of stained tissue sections were acquired using a DAGE-330 color camera and captured with a Pentium P-166 IBM compatible computer. The digitized images were converted to a gray scale and analyzed using Image-Pro plus software (Media Cybernetics), according to Mize’s study [13], as described before [6]. To eliminate variation, the microscope light source intensity used during image capture was kept constant for all sections stained on a given day.

Statistics

ANOVA was employed to assess statistical differences between all experimental groups tested. Multiple com-
The efficacy of 1,25(OH)2D3 and 19-Nor-1,25(OH)2D2 in preventing high P-induced PT hyperplasia in uremic rats was determined seven days after the induction of renal insufficiency by 5/6 nephrectomy. Preliminary studies defined minimal antiproliferative, non-hypercalcemic doses of the two vitamin D metabolites employed in the present protocols. To compare the mechanisms mediating the control of PT growth by vitamin D and high dietary calcium, we used a diet containing 2.0% Ca to arrest uremia-induced PT hyperplasia and 1.2% P to prevent hypophosphatemia.

**RESULTS**

Table 1 shows serum chemistries in normal and uremic rats for all the experimental condition tested. Serum creatinine increased similarly in all uremic animals regardless of dietary P or Ca intake. Serum P levels increased in all uremic groups compared to normal controls. In uremic rats fed the high P diet, treatment with either 1,25(OH)2D3 or 19-Nor-1,25(OH)2D2 did not further increase serum P levels. At the doses tested, there was no significant difference in serum P between 1,25(OH)2D3 and 19-Nor-1,25(OH)2D2 treated animals. As expected, serum P was lower in the uremic rats fed a 0.2% P diet compared to those fed a high P diet. Treatment with either vitamin D metabolite did not increase ionized Ca above the levels of normal rats fed the same high P diet. Only in uremic rats fed a high Ca diet was ionized Ca higher than normal controls.

**Effects of vitamin D treatment on serum PTH**

Figure 1A depicts serum PTH levels in each experimental condition. Uremic rats fed the high P diet had much higher serum PTH than normal rats fed the same diet. Both 1,25(OH)2D3 and 19-Nor-1,25(OH)2D2 prevented the increase in serum parathyroid hormone induced by high dietary phosphorus. At day 7 after 5/6 nephrectomy, the control of serum PTH by either vitamin D treatment in the rats fed a high P diet, was similar to that exerted by P restriction. 1,25(OH)2D3, 19-Nor-1,25(OH)2D2 and P restriction prevented uremia-induced elevations in serum PTH with no increases in ionized Ca levels.
Effects of vitamin D treatment on parathyroid gland growth

There was no contaminating thyroid tissue surrounding the parathyroid gland used for weight determinations as demonstrated in the immunohistochemistry sections. Figure 1B shows the effects of vitamin D treatment on PT gland weight. In uremic rats fed high P diet, the weight of the PT glands was higher than in normal controls fed the same diet. Both 1,25(OH)₂D₃ and 19-Nor-1,25(OH)₂D₂ prevented the enhancement of PT gland weight observed in uremic rats fed a high P diet. The growth arrest induced by vitamin D treatment counteracting uremia and high dietary P was similar to that achieved by phosphate restriction in PT glands in which renal failure was the only growth promoting signal.

To directly measure PT-cell proliferation rates, two markers of mitotic activity, Ki67 (cell cycle-associated nuclear antigen) and PCNA (proliferating cell nuclear antigen), were examined. The number of positive cells per parathyroid gland area is shown in Figure 2. Higher levels of both PCNA and Ki67 in PT glands from uremic rats fed a high phosphorus diet compared to normal controls. Lower PT-Ki67 and PT-PCNA expression was observed after seven days in uremic rats fed high P and treated with either 1,25(OH)₂D₃ or 19-Nor-1,25(OH)₂D₂. At day 7 after the onset of uremia, mitotic activity in rats fed high P and treated with either vitamin D metabolite was as low as that observed in P-restricted animals. The higher levels of PCNA compared to Ki67 in each experimental group reflect the prolonged half-life of PCNA compared to Ki67 after completion of mitosis.

Effects of vitamin D treatment on parathyroid p21 expression

To identify the mechanisms mediating the antiproliferative properties of vitamin D treatment, we first examined immunohistochemical staining for p21 in PT glands from rats of each experimental condition. The mixed cytosolic/nuclear p21 distribution precludes accurate measurement of p21 expression using Image-Pro Plus. Vitamin D treatment with either 1,25(OH)₂D₃ or 19-Nor-1,25(OH)₂D₂ markedly induced PT-p21 expression in uremic rats fed a high P diet (Fig. 3). The induction of PT p21 by 1,25(OH)₂D₃ or 19-Nor-1,25(OH)₂D₂ (grade ++ + ) was lower than that achieved with phosphate restriction (grade + + + ), but higher than PT p21 expression in uremic rats fed a high P diet (grade + ).

Effects of vitamin D treatment on parathyroid TGF-α expression

Immunohistochemical assessment of PT TGF-α levels was performed using a monoclonal antibody that recognizes all TGF-α isoforms. As expected, the highest immunoreactivity for TGF-α was found in the PT glands from uremic rats fed a high P diet (Table 2). Vitamin D treatment prevented the increases in PT TGF-α induced by uremia and high dietary P. The efficacy of 1,25(OH)₂D₃ and 19-Nor-1,25(OH)₂D₂ in preventing increases in TGF-α was similar to that of P restriction (Fig. 4).

The findings of vitamin D control of TGF-α expression were confirmed in a model of chronic renal failure (Fig. 5). Normal and 5/6 nephrectomized rats were fed a high P diet. Uremic rats received either vehicle or 100 ng of 19-Nor-1,25(OH)₂D₂ three times weekly for 60 days after the onset of uremia. 19-Nor-1,25(OH)₂D₂ ameliorated the increase in PT TGF-α induced by chronic renal failure and high dietary P (Fig. 5).

Effects of high dietary calcium on serum PTH, parathyroid gland growth, and parathyroid p21 and TGF-α expression

High dietary Ca, a well-known promoter of PT growth arrest in renal failure, prevented both the increase in
Fig. 3. Effects of vitamin D treatment and high dietary Ca on PT p21 expression. Representative photomicrographs of immunohistochemical staining of p21 in PT tissue of normal and 5/6 nephrectomized rats undergoing one of the following experimental protocols at day 7 after the onset of renal failure: normal + high P (N-HP) diet; uremic (U) + HP diet + vehicle (U-HP), 1,25D (4 ng/day) (U-HP + 1,25D), or 19-Nor (30 ng/day) (U-HP + 19-Nor); U + low P diet (U-LP); U + high calcium diet (U-HCa). Magnification ×400.

Table 2. Semiquantitative analysis of parathyroid (PT) transforming growth factor-α (TGF-α) expression

<table>
<thead>
<tr>
<th></th>
<th>TGF-α IOD/area</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-HP</td>
<td>0.013 ± 0.004a</td>
</tr>
<tr>
<td>U-HP</td>
<td>0.035 ± 0.004</td>
</tr>
<tr>
<td>U-HP + 1,25D</td>
<td>0.009 ± 0.001b</td>
</tr>
<tr>
<td>U-HP + 19-Nor</td>
<td>0.015 ± 0.001</td>
</tr>
<tr>
<td>U-LP</td>
<td>0.007 ± 0.001a</td>
</tr>
<tr>
<td>U-HCa</td>
<td>0.016 ± 0.005a</td>
</tr>
</tbody>
</table>

Values for TGF-α represent the mean ± SEM from at least 6 rats per group. Abbreviations are in Table 1.

Figs. 3. Effects of vitamin D treatment and high dietary Ca on PT p21 expression. Representative photomicrographs of immunohistochemical staining of p21 in PT tissue of normal and 5/6 nephrectomized rats undergoing one of the following experimental protocols at day 7 after the onset of renal failure: normal + high P (N-HP) diet; uremic (U) + HP diet + vehicle (U-HP), 1,25D (4 ng/day) (U-HP + 1,25D), or 19-Nor (30 ng/day) (U-HP + 19-Nor); U + low P diet (U-LP); U + high calcium diet (U-HCa). Magnification ×400.

Table 2. Semiquantitative analysis of parathyroid (PT) transforming growth factor-α (TGF-α) expression

<table>
<thead>
<tr>
<th></th>
<th>TGF-α IOD/area</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-HP</td>
<td>0.013 ± 0.004a</td>
</tr>
<tr>
<td>U-HP</td>
<td>0.035 ± 0.004</td>
</tr>
<tr>
<td>U-HP + 1,25D</td>
<td>0.009 ± 0.001b</td>
</tr>
<tr>
<td>U-HP + 19-Nor</td>
<td>0.015 ± 0.001</td>
</tr>
<tr>
<td>U-LP</td>
<td>0.007 ± 0.001a</td>
</tr>
<tr>
<td>U-HCa</td>
<td>0.016 ± 0.005a</td>
</tr>
</tbody>
</table>

Values for TGF-α represent the mean ± SEM from at least 6 rats per group. Abbreviations are in Table 1.

DISCUSSION

These studies demonstrate that the induction of parathyroid p21 and the prevention of increases in TGF-α are common mediators of the suppression of uremia and high phosphorus-induced parathyroid hyperplasia by vitamin D treatment and high dietary calcium.

The molecular mechanisms responsible for uremia induction of PT cell growth are still incompletely understood [3, 14, 15]. Different groups have demonstrated an association between changes in the content of cell cycle regulators and PT hyperplasia in renal failure. Vasef et al [16] showed an overexpression of cyclin D1 protein in human hyperplastic PT glands, while Erickson et al [17] described a reduction of the cyclin-dependent kinase inhibitor p27kip1 protein expression in human PT hyperplasia compared to normal PT glands.

In experimental models of renal failure in rats, enhanced PT cell proliferation rather than hypertrophy causes PT gland enlargement [18]. Most PT gland growth occurs within four to six days of the onset of renal failure [19, 20]. Studies to identify potential mechanisms for dietary P regulation of PT cell growth in early uremia demonstrated that high P-induced PT hyperplasia is associated with increased expression of the growth promoter TGF-α [6]. In contrast, PT growth arrest induced by low dietary P is associated with increased PT expression of p21 and occurred in the absence of changes in serum 1,25(OH)2D3, a direct transcriptional activator of the p21 gene. Dietary P-induced changes in both p21 and TGF-α expression are specific for the PT gland [6].
Fig. 4. Effects of vitamin D treatment and high dietary Ca on PT TGF-α expression. Representative photomicrographs of immunohistochemical staining of TGF-α in PT tissue of normal and 5/6 nephrectomized rats undergoing one of the following experimental protocols at day 7 after the onset of renal failure: normal + high P (N-HP) diet; uremic (U) + HP diet + vehicle (U-HP), 1,25D (4 ng/day) (U-HP + 1,25D), or 19-Nor (30 ng/day) (U-HP + 19Nor); U + low P diet (U-LP); U + high calcium diet (U-HCa). Magnification ×100.

Fig. 5. Effects of 19-Nor-1,25(OH)2D3 treatment on PT TGF-α expression in chronic renal failure. Representative photomicrographs of immunohistochemical staining of TGF-α in PT tissue of normal and 5/6 nephrectomized rats undergoing one of the following experimental protocols at day 60 after the onset of renal failure: normal + high P (N-HP) diet; uremic (U) + HP diet + vehicle (U-HP); 19-Nor (100 ng 3×/week) (U-HP + 19-Nor). Magnification ×100.
Since vitamin D suppresses PT cell proliferation in vitro [7] and in vivo in uremic rats [8], the present studies specifically addressed the efficacy of vitamin D therapy (1,25-dihydroxyvitamin D$_3$ and the analog 19-Nor-1,25-dihydroxyvitamin D$_2$) to counteract the PT hyperplasia induced by high dietary P in early uremia.

Pilot studies demonstrated that doses of 4 ng of 1,25(OH)$_2$D$_3$ and 30 ng of 19-Nor-1,25(OH)$_2$D$_2$ were effective in controlling PT hyperplasia with no hypercalcemic or hyperphosphatemic effects. At these doses, both vitamin D compounds not only prevented the increase in the PTH levels, but also ameliorated the enlargement of the PT glands induced by uremia and worsened by high dietary P. The efficacy of 1,25(OH)$_2$D$_3$ and 19-Nor-1,25(OH)$_2$D$_2$ in controlling both serum PTH and PT gland size was similar to that described for P restriction in the uremic rat model [6], which was utilized in the present protocols as a positive control of PT growth arrest. Further support for vitamin D control of high P-induced PT gland growth was provided by immunohistochemical staining for two markers of mitotic activity: Ki67 and PCNA. Both 1,25(OH)$_2$D$_3$ and 19-Nor-1,25(OH)$_2$D$_2$ prevented the marked increase in both PT Ki67 and PT PCNA expression observed in uremic rats fed the same high P diet. The higher PCNA staining compared to Ki67 could be the result of the longer half-life of PCNA, which allows immunohistochemical detection of PCNA for a considerable time after completion of mitosis [21]. Similar to studies in several carcinomas [22], our studies revealed that Ki67 is a more accurate marker of PT cell proliferation in paraffin embedded tissues than PCNA. Similar low levels of both markers of cell proliferation were present in uremic rats fed low P or high Ca, two dietary maneuvers effective in preventing PT gland enlargement.

Liu et al demonstrated that increases in p21 mediate the antiproliferative effects of 1,25(OH)$_2$D$_3$ in human monocytes [9]. These authors showed that direct activation of p21-gene transcription was sufficient for 1,25(OH)$_2$D$_3$ to suppress growth in the human myelomonocytic cell line U937 [9]. The present studies confirm that vitamin D treatment also induces the expression of p21 protein in rat PT glands. Further support for these findings came from studies in patients with secondary hyperparathyroidism (abstract; Tokumoto et al, J Am Soc Nephrol 11: 584A, 2000). A strong correlation was found between the more aggressive nodular form of PT growth and reduced p21 expression. In these patients, the lower PT p21 content, the lower the expression of the vitamin D receptor. This finding suggests that 1,25(OH)$_2$D$_3$ induction of PT p21 also involves enhancement of p21 gene transcription by the 1,25(OH)$_2$D$_3$-vitamin D receptor complex. Clearly, 1,25(OH)$_2$D$_3$ and 19-Nor-1,25(OH)$_2$D$_2$ induction of PT p21 expression could partially account for the antiproliferative effects of both sterols on high P-induced PT hyperplasia.

In contrast to the antiproliferative properties of enhanced PT p21 expression, increases in TGF-α content act as a growth promoter in secondary hyperparathyroidism [23]. Studies in early uremia demonstrating that high dietary P may induce PT cell proliferation by increasing TGF-α expression [6], led us to examine the effects of vitamin D treatment on PT TGF-α levels. As expected, the highest immunoreactivity for TGF-α was found in the PT glands from uremic rats fed a high P diet. Surprisingly, both 1,25(OH)$_2$D$_3$ and 19-Nor-1,25(OH)$_2$D$_2$ prevented the increases in PT TGF-α induced by uremia and high dietary P. Clearly, in this early uremia model of high P-induced PT hyperplasia, there is a direct temporal correlation between increases in PT TGF-α expression and the markers of mitotic activity Ki67 and PCNA. In contrast, the inhibition of PT gland growth by vitamin D therapy associates not only with the induction of PT p21, but also with the prevention of the increases in TGF-α. Furthermore, 19-Nor-1,25(OH)$_2$D$_2$-control of PT gland growth in a model of chronic renal failure also could be partially accounted for the analog preventing the increases in TGF-α expression induced by high dietary P. This is a novel finding suggesting that the control of PT-TGF-α expression by vitamin D treatment could mediate the antiproliferative properties of 1,25(OH)$_2$D$_3$ and 19-Nor-1,25(OH)$_2$D$_2$ on high P induced-PT hyperplasia in renal failure. Although a computer analysis of the rat TGF-α promoter indicated no consensus vitamin D responsive sequences (VDRE), several mechanisms could mediate 1,25D transcriptional expression of TGF-α. Alternative mechanisms for the suppressive effects of TGF-α expression by the 1,25D-vitamin D receptor complex include: (a) a non classical VDRE [24]; (b) a ligand activated VDR preventing the binding of a transcriptional activator of the TGF-α gene [25, 26] or indirect effects through induction of intermediary TGF-α repressor proteins [27]. Identification of the mechanisms for vitamin D inhibition of TGF-α expression demands further investigation.

Studies in psoriasis, a hyperproliferative disorder of the skin, provide some indirect evidence of the impact of down-regulation of TGF-α expression by vitamin D treatment in growth arrest. TGF-α regulates the growth of normal and neoplastic cells in various tissues. TGF-α mRNA and protein are expressed in human keratinocytes [28]. TGF-α expression in psoriatic epidermis is higher than in normal skin [29]. In view of the efficacy of 1,25(OH)$_2$D$_3$ and its less calcemic analogs in the treatment of psoriasis [30, 31], as well as our findings in uremic PT growth, it is possible that 1,25(OH)$_2$D$_3$ and vitamin D analogs arrest psoriatic keratinocyte proliferation by preventing increases in TGF-α expression. In fact, preliminary studies in our laboratory using the human keratinocyte cell line A431, which mimic psoriatic keratinocytes in overexpressing TGF-α, demonstrate that the
suppression of growth induced by 1.25D treatment is associated with two distinct cytotoxic leukemia cell lines (UF-1) associated with expression of p21 and p27. Treatment by vitamin D3 suppresses growth induced by 1,25D treatment. High dietary Ca controlled uremia-induced PT hyperplasia as demonstrated by both a reduction of PT gland size and PT expression of the two markers of mitotic activity Ki67 and PCNA. Similar to vitamin D treatment, high dietary Ca enhanced PT p21 content and prevented the increases in PT TGF-α induced by uremia. The mechanisms for the regulation of PT p21 and TGF-α expression by high dietary Ca are different from those elicited by vitamin D treatment. In fact, in the vitamin D receptor-ablated mice, a Ca-enriched diet also prevents the development of PT hyperplasia in both hypocalcemic and normocalcemic states [32]. The recent association found between hypercalcemia and low plasma levels of TGF-α in cancer patients suggests a systemic control of TGF-α expression by calcium [33]. In vitamin D therapy, it also is possible that 1.25D-mediated increases in intracellular Ca could contribute further to enhance p21 expression and the unknown mechanisms mediating down-regulation of TGF-α.

In summary, both 1,25-dihydroxyvitamin D3 and 19-Nor-1,25-dihydroxyvitamin D2 are effective in preventing the parathyroid hyperplasia induced by renal failure and further enhanced by high dietary phosphorus. The inhibition of high phosphorus-induced parathyroid growth by vitamin D treatment is associated with two distinct pathways: one is the expected, but not previously assessed, induction of parathyroid p21 content. The second is the novel finding of the ability of vitamin D treatment to prevent the increases in PT TGF-α induced by renal failure and worsened by high dietary P.

Similar to vitamin D treatment, high dietary Ca inhibited PT hyperplasia by enhancing PT-p21 content and preventing the increases in PT TGF-α induced by uremia. Clearly, regulation of parathyroid expression of p21 and TGF-α could represent a key target for therapeutic interventions in controlling PT hyperplasia in renal failure.

ACKNOWLEDGMENTS

The Editor is grateful to Jared Grantham, who served as Guest Editor during the review of this manuscript. This research was supported in part by a grant from Abbott Pharmaceuticals. Part of these studies were presented at the American Society of Nephrology 2000 Meeting in Toronto (abstract A3030; J Am Soc Nephrol 11: 574A, 2000). The authors thank Ms. Sue King for performing blood chemistries, Ms. Patricia Clay for measuring rat PTH, and Dr. Diego Brancaccio and Dr. Maurizio Gallieni for helpful discussions.

REFERENCES