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1,25-Dihydroxyvitamin D downregulation of TGFα/EGFR expression and growth signaling: a mechanism for the antiproliferative actions of the sterol in parathyroid hyperplasia of renal failure☆

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Abstract

Elevated serum levels of parathyroid hormone (PTH) contribute to the increased morbidity and mortality in renal failure patients. Parathyroid gland hyperplasia is a major cause of high serum PTH. The present studies used the rat model of renal failure to address the mechanisms underlying uremia-induced parathyroid hyperplasia and the antiproliferative properties of vitamin D therapy (1,25-dihydroxyvitamin D ($1,25(OH)_2D_3$) or its less calcemic analogs). Enhanced TGFα/EGFR co-expression is the major mitogenic signal in uremic parathyroid glands. At early stages of renal failure, vitamin D therapy efficiently counteracts uremia- and high phosphorus-induced hyperplasia by inhibiting the increases in parathyroid-TGFα/EGFR co-expression. In established hyperparathyroidism, characterized by highly enhanced-TGFα/EGFR co-expression, vitamin D therapy arrests growth by suppressing EGFR-growth signals from the plasma membrane and nuclear EGFR actions as a transactivator of the cyclin D1 gene, an important contributor to parathyroid hyperplasia in humans. In advanced renal failure, reduced-parathyroid vitamin D receptor levels limits the antiproliferative efficacy of vitamin D therapy. However, non-antiproliferative doses of 1,25-dihydroxyvitamin D enhance the anti-EGFR actions of EGFR–tyrosine kinase inhibitors (TKI). In fact, combined 1,25-dihydroxyvitamin D/TKI therapy inhibits parathyroid hyperplasia more efficiently than phosphorus restriction, the most powerful promoter of parathyroid growth arrest available at present.

Keywords: Parathyroid hyperplasia; TGFα; EGFR; Renal failure

1. Introduction

Secondary hyperparathyroidism is a frequent complication of chronic renal failure characterized by parathyroid hyperplasia and enhanced synthesis and secretion of parathyroid hormone (PTH) [1–3]. High circulating PTH levels causes osteitis fibrosa and bone loss, typical features of renal osteodystrophy, as well as cardiovascular complications, which increase mortality in renal failure patients [4–6].

The three main direct causes of hyperparathyroidism are hypocalcemia, hyperphosphatemia, and 1,25-dihydroxyvitamin D ($1,25(OH)_2D_3$) deficiency. Hyperphosphatemia and $1,25(OH)_2D_3$ deficiency also enhance parathyroid function indirectly by lowering serum calcium (Ca) [1–3,7]. As renal disease progresses, a reduction in parathyroid content of the Ca sensing receptor (CaSR) and the vitamin D receptor (VDR) renders the parathyroid glands more resistant to suppression of cell growth and PTH synthesis in response to Ca and $1,25(OH)_2D_3$ [8–10]. Lack of an appropriate parathyroid cell line and the rapid de-differentiation of primary cultures of parathyroid cells have impeded direct characterization of the pathogenic mechanisms underlying uremia induced parathyroid hyperplasia. To address these issues, we used the only experimental approach available at present, the 5/6 nephrectomized rat model. The results of these studies have implicated increases in parathyroid co-expression of the growth promoter TGFα and its receptor, the EGFR, in uremia-induced enhancement of proliferative activity and gland size [11,12]. In addition, they have revealed novel insights into the antiproliferative actions of vitamin D therapy in arresting growth driven by overexpressed EGFR. This review present the most recent experimental evidence on the relevance of $1,25(OH)_2D_3$ downregulation of the autocrine TGFα/EGFR growth loop in the control of parathyroid hyperplasia in renal failure.

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2. Enhancement of TFGα/EGFR co-expression is an early event in parathyroid hyperplasia of renal failure

Similar to secondary hyperparathyroidism in humans [13], TGFα expression is higher in uremic rats compared to normal controls [11]. More importantly, when uremia-induced parathyroid hyperplasia is worsened by a high P [11] or low Ca intake [14], there is a temporal relationship between increases in parathyroid TGFα, the enlargement of the parathyroid glands, and proliferating activity. Furthermore, suppression of uremia-induced parathyroid hyperplasia by high dietary Ca (2% Ca diet) or P restriction prevented the increases in parathyroid TGFα induced by the onset of renal failure by day 7 after 5/6 nephrectomy [11,12]. These findings suggest a role for TGFα as a mitogenic stimulus in the parathyroid glands, triggered by uremia and further enhanced by high dietary P [11] or low Ca intake [14].

Since enhanced co-expression of TGFα and EGFR associates with more aggressive growth in normal and transformed tissues [15], we examined whether uremia and dietary Ca and P manipulations also modulate parathyroid EGFR expression. Quantification of TGFα and EGFR immunostaining, as previously described [11], demonstrated an average increase of 2.3-fold above normal by high dietary P or low Ca intake by 7 days after the onset of uremia. Conversely, similar to their efficacy in preventing increases in parathyroid TGFα content, high dietary Ca and P restriction prevented uremia-induced increases in EGFR content. The strong association between enhanced TGFα/EGFR co-expression and high proliferative activity does not constitute substantial evidence of its contribution to uremia induced parathyroid hyperplasia. To directly address the pathophysiological relevance of enhanced TGFα/EGFR co-expression on parathyroid-cell growth, we utilized the EGFR–tyrosine kinase inhibitor AG1478, effective in inhibiting the autocrine TGFα/EGFR-growth loop in vitro [16] and in vivo [17,18].

3. Enhanced co-expression of TGFα and EGFR is a major pathogenic mechanism for parathyroid hyperplasia

Similar to most EGFR–tyrosine kinase inhibitors (TKIs), AG1478 is a small molecule, highly selective for EGFR-tyrosine kinase [19], that competes with ATP binding and reversibly inhibits tyrosine *trans*-phosphorylation, thereby blocking downstream signaling.

5/6 nephrectomized rats (180–200 g body weight), fed a high P diet (1.2% P), received intraperitoneal injections of either vehicle (1 ml of DMSO:PBS, 1:1) or AG1478 (25 mg/kg body weight, every other day for 1 week). This dose is half of that effective to arrest the growth of aggressively growing tumors in mice (given daily) and was further adjusted considering a metabolic mouse:rat ratio of 2. This dose had no adverse effect as judged by no differences in body weight, serum creatinine, BUN, and pH. Ionized Ca, total Ca, and P levels were similar in the uremic control group and the AG1478-treated animals. AG1478 reduced the enlargement of the parathyroid glands [20] and the proliferative activity by 60% compared to uremic high P-controls. These findings demonstrate that enhanced TGFα/EGFR co-expression is a main contributor to uremia-induced parathyroid hyperplasia.

4. The antiproliferative properties of prophylactic vitamin D therapy in the parathyroid glands involve downregulation of TGFα/EGFR co-expression

The efficacy of prophylactic administration of either 1,25-dihydroxyvitamin D or the less calcemic vitamin D analog 19-Nor-1,25$(OH)_2D_2$ (at doses of 4 or 30 ng, respectively) to arrest parathyroid cell growth and consequently, the enlargement of the parathyroid glands induced by uremia and high dietary P, associates with prevention of the increases in TGFα [11,12]and EGFR expression (Fig. 1). Several reports demonstrate a direct and cell specific regulation of EGFR mRNA by 1,25-dihydroxyvitamin D [21,22]. Since TGFα activation of EGFR induces both TGFα-and EGFR-gene expression [23,24], it is also possible that 1,25$(OH)_2D_3$ inhibition of EGFR activation [25] mediates the suppressive effects of the sterol on TGFα and EGFR expression.

5. 1,25$(OH)_2D_3$ antiproliferative properties in EGFR overexpressing cells involve downregulation of both classical EGFR-growth signals from the cell membrane and EGFR-transactivation of the cyclin D1 gene

The efficacy of vitamin D therapy to arrest TGFα-driven hyperplastic growth in established secondary hyperparathyroidism and psoriasis [26,27] suggests that 1,25$(OH)_2D_3$ could downregulate TGFα/EGFR-growth signals.

Recent studies from our laboratory [25] in the human epidermoid carcinoma cell line A431, overexpressing TGFα and EGFR [28,29], and in NR6 cells, normal human embryonic cells overexpressing exclusively EGFR, demonstrate that 1,25$(OH)_2D_3$ treatment reduced growth by decreasing EGFR activation. In A431 cells, the main mechanism involved in 1,25D-inhibition of EGFR activation is induction of EGFR accumulation in early endosomes by prevention of its recycling to the plasma membrane [25]. As a consequence, there is reduced membrane bound EGFR and a marked reduction in EGFR activation by tyrosine phosphorylation upon ligand binding. The ability of 1,25$(OH)_2D_3$ to inhibit autocrine EGFR activation in EGFR-overexpressing cells results in reduced phosphorylation of ERK1/2, the main downstream growth signal. In aggressively growing tumors driven by enhanced TGFα/EGFR co-expression, prevention of nuclear P-ERK1/2 translocation is used as an index of

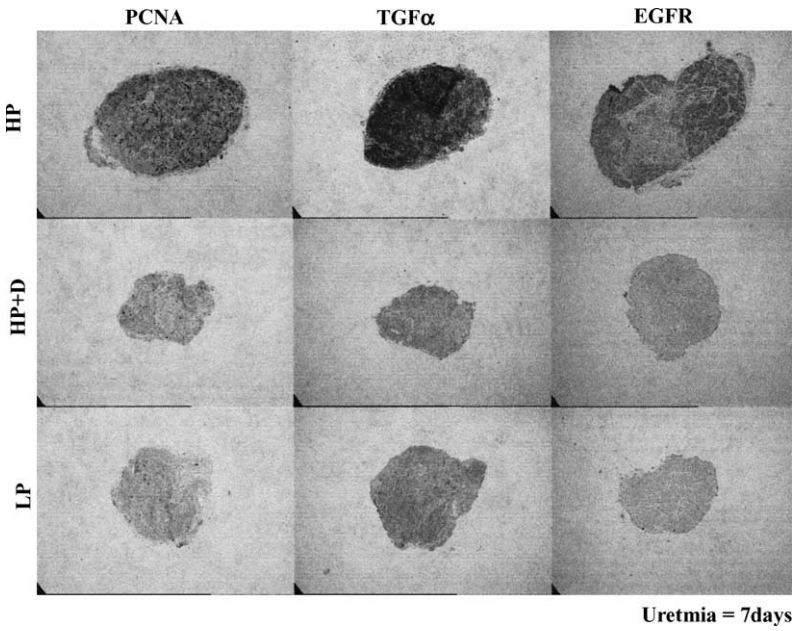

Fig. 1. Enhanced parathyroid co-expression of TGFα and EGFR correlates with high proliferative activity (PCNA). Representative photomicrographs of immunohistochemical staining of parathyroid tissue from 5/6 nephrectomized rats fed a high P (0.9% P) (HP) or a low P (0.2% P) (LP) diet, which received either vehicle or vitamin D therapy (D) (4 ng $1,25(OH)_2D_3$ or 30 ng 19-nor$(OH)_2D_2$) every other day for 1 week. Magnification 100×.

the efficacy of anti-EGFR therapy [17]. The potency of $1,25(OH)_2D_3$ to downregulate TGFα/EGFR autocrine signals in A431 cells is similar to that of EGFR–tyrosine kinase inhibitors as shown by the lack in nuclear translocation of phosphorylated ERK1/2 in A431 cells treated with $1,25(OH)_2D_3$ [25].

In A431 cells, $1,25(OH)_2D_3$ inhibits classical EGFR-growth signals from the plasma membrane, common to most tyrosine-kinase receptors, and the novel EGFR nuclear actions as a transactivator (co-activator) of the cyclin D1 gene, an important contributor to parathyroid hyperplasia in humans. The mechanism appears to involve impaired nuclear translocation of the EGFR, possibly as a consequence of the stasis of the receptor in the endocytic compartment, since total EGFR levels are not affected by $1,25(OH)_2D_3$ treatment [26].

6. $1,25(OH)_2D_3$ potentiates the growth arrest induced by TKI in EGFR-overexpressing cells

Carcinogenic cells overexpressing EGFR subvert the G1 to S transition by decreasing the levels of cyclin-dependent kinase inhibitor p27 and inducing cyclin D1, the two cell cycle regulators more frequently associated with high proliferating activity in the parathyroid glands of renal failure patients. Reports on the mechanisms mediating the inhibition of EGFR-driven growth by AG1478 demonstrate that upregulation of p27 protein levels is mandatory for the efficacy of TKI therapy [17,30]. Since $1,25(OH)_2D_3$ induces p21 and p27 expression in several tissues including the parathyroid glands [11,31] and reduces cyclin D1 protein and mRNA levels in colon carcinoma [22] we examined potential synergistic effects of the sterol on TKI therapy.

Dose response studies on the efficacy of EGFR–TKI to suppress A431 growth demonstrated that maximal inhibition was achieved after a 20 h exposure to 1–3 μM AG1478. Simultaneous treatment with submaximal (0.1 μM) and maximal inhibitory (1 μM) doses of AG1478 and 100 nM $1,25(OH)_2D_3$ resulted in an additional suppression of the growth induced by AG1478 alone, as shown by the reduction in thymidine incorporation to DNA in Table 1.

These results demonstrate that mechanisms unrelated to inhibition of EGFR-growth signals also contribute to $1,25(OH)_2D_3$ suppression of growth in EGFR-overexpressing cells. They also suggest the potential benefits of combined therapy with TKIs and $1,25(OH)_2D_3$ vitamin D in the control of hyperplastic growth driven by EGFR overexpression. We examined the persistence of the observed synergistic/additive effects in established hyperparathyroidism. Sprague-Dawley rats (250 g body weight) were 5/6

Table 1
$1,25(OH)_2D_3$ potentiates the antiproliferative actions of the EGFR-TKI AG1478

Control	88381 ±2027
1,25D (100 nM)	62593 ± 1045
AG (0.1 μM)	32478 ± 2737
AG + 1,25D	18222 ± 539
AG (1 μM)	8073 ± 437
AG + 1,25D	5341 ± 179

[3]H-thymidine incorporated to DNA by A431 cells untreated or after a 20 h treatment with $1,25(OH)_2D_3$ (1,25D), AG1478 (AG) or combination. Results represent the mean ± SEM of five determinations per experimental condition.

Table 2
$1,25(OH)_2D_3$ potentiates the antiproliferative actions of the EGFR-TKI inhibitor Tarceva in established experimental hyperparathyroidism

	PTG weight (μg)
LP	395.2 ± 31.5 (11)
HP + vehicle	527.2 ± 46.7 (13)
HP + 1,25D	520.8 ± 44.2 (9)
HP + Tarceva	421.0 ± 29.4 (17)
HP + Tarceva + 1,25D	335.5 ± 39.8 (13)

Parathyroid gland weight of glands from 5/6 nephrectomized rats fed a high P (0.9% P) diet for 2 weeks followed by two additional weeks of low dietary P (LP) or high dietary P (0.9%) plus vehicle; 4 ng $1,25(OH)_2D_3$ every other day (1,25D); Tarceva (6 mg/kg body weight daily) or combination therapy. Values represent the mean ± SEM of (*n*) parathyroid glands.

nephrectomized, fed a high P diet (0.9% P) for 2 weeks, and treated for two additional weeks as follows: (a) high P diet (HP) + vehicle; (b) low P diet (0.2% P); (c) HP + TKI (Erlotinib (Tarceva, Genentech, Inc.), 6 mg/kg bw, daily, ip); (d) HP + 1,25D (4 ng, every other day, ip); (e) HP + Erlotinib + 1,25D, at the doses indicated above.

Table 2 shows that 2 weeks of P restriction, known to arrest PT hyperplasia in established 2HPT, reduced by 23.4% the enlargement of the parathyroid glands observed in the HP controls.

There were no changes in serum creatinine, ionized Ca, or P with treatment with either $1,25(OH)_2D_3$, Erlotinib, or $1,25(OH)_2D_3$ + Erlotinib. Treatment with Erlotinib inhibited parathyroid hyperplasia by 21.9%, an efficacy similar to that of P restriction. The latter results further support the important contribution of growth promoting signals from overexpressed TGFα/EGFR in established secondary hyperparathyroidism. Due to the vitamin D resistance associated to established hyperparathyroidism, the 4 ng dose of $1,25(OH)_2D_3$, antiproliferative when given prophylactically in early uremia, was ineffective to prevent the enlargement of the PTG induced by uremia and high dietary P. However, at this low dose, $1,25(OH)_2D_3$ potentiated the antiproliferative effects of Erlotinib's to induce an inhibition of parathyroid gland enlargement of 36.8%, more potent than that of P restriction. These results suggest that the combination of $1,25(OH)_2D_3$ at non-antiproliferative doses plus Erlotinib is a novel and highly effective therapeutic tool in controlling hyperplasia in established hyperparathyroidism, in which vitamin D resistance limits the efficacy of exclusive therapy with $1,25(OH)_2D_3$ or its less calcemic analogs.

7. Concluding remarks

The use of the rat model of experimental renal failure and anti-EGFR therapy with small molecule EGFR–TKIs has rendered conclusive evidence of the major contribution of the autocrine TGFα/EGFR growth loop to parathyroid hyperplasia in renal failure. Consequently, they have also revealed the importance of $1,25(OH)_2D_3$ inhibition of TGFα/EGFR expression and growth signals for the efficacy of vitamin D therapy in secondary hyperparathyroidism of renal failure. More importantly, they have shown the ability of non-antiproliferative doses of $1,25(OH)_2D_3$ to enhance the potent growth inhibition by EGFR-TKIs in growth driven by overexpressed EGFR in vitro and in vivo.

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