A. PTH

It has been suggested that HDM is caused by raised levels of PTH associated with secondary hyperparathyroidism, rather vitamin D deficiency itself [91]. Fatigue and muscular weakness are classically associated with primary hyperparathyroidism [92–95], and successful parathyroid surgery results in significant improvement of these muscular symptoms [92,94,96]. In addition, muscle biopsies obtained from patients with primary hyperparathyroidism reveal type II fiber atrophy, very much like the changes seen in HDM. Treatment with PTH reduces the intracellular content of inorganic phosphate, creatine phosphate, and CaATPase [97], which are exactly the findings we reported after analysis of muscle biopsies and ^{31}P-MR-spectroscopy in patients with HDM [23,66]. Furthermore, mitochondrial oxygen consumption and the activity of creatine phosphokinase and CaATPase are reduced and oxidation of long-chain fatty acids impaired by PTH [98].

Thus, many parallels exist between myopathy caused by primary hyperparathyroidism and HDM. However, muscle weakness is not always present in patients with primary hyperparathyroidism [3,99], and HDM can be present despite normal PTH levels [9]. Further, improvements in muscle strength after surgery for primary hyperparathyroidism do not correlate to postoperative decreases in PTH or calcium [94,100].

If PTH was the only effector on skeletal muscle, reversal of secondary hyperparathyroidism by a high intake of calcium should reverse the muscular symptoms. However, in their rat study Rodman and Baker [45] detected severe perturbations of muscle function in vitamin D-deficient rats despite high serum levels of calcium and phosphate (Fig. 4). Furthermore, in a study on type II fiber size in hip fracture patients, Sato *et al.* [54] reported a significant correlation between serum levels of 25OHD and fiber size—no correlation to PTH was reported.

Generally, clinical studies [8,56,89] report inverse correlations between PTH and muscle symptoms, and positive correlations to 25OHD [8,56,58,59,89]. In a comment accompanying the study of Stein *et al.* [56], Birge [83] suggested that PTH might be a better biological marker for vitamin D deficiency at the tissue level than serum levels of 25OHD, and this could be the reason why PTH and not 25OHD come out as significant determinants in multiple regression analyses. In this context, however, the significant interaction between 25OHD and PTH has to be taken into account.

In conclusion: There is strong evidence for a direct effect of vitamin D on muscle both in clinical and experimental studies. It is possible, however, that secondary hyperparathyroidism may exert additive or synergistic effects on HDM development.

B. 1,25(OH)$_2$D

Theoretically, 1,25(OH)$_2$D concentrations should be the most likely effector of vitamin D effects on muscle—and indeed, a large amount of experimental data support this notion (see Chapter 55). In clinical studies, however, this expected relationship finds less support [59]. Glerup *et al.* [8,23] found no correlation between muscle function and 1,25(OH)$_2$D ($r = -0.14$, NS), whereas maximal knee extension strength correlated significantly to 25OHD ($r = 0.34$, $p < 0.01$). In fact, it is common to see severe symptoms of HDM with normal or even elevated values of 1,25(OH)$_2$D. Furthermore, hypovitaminosis D–related symptoms (diffuse muscle pain, deep bone pain, paresthesia, fatigue, muscle cramps, joint pain) all correlated to 25OHD (Kruskal-Wallis ANOVA: $p < 0.001$) but not to 1,25(OH)$_2$D (NS). The absence of correlations to 1,25(OH)$_2$D is probably explained by several factors. First, renal 1-α-hydroxylase activity is under tight control by PTH levels, resulting in normal or even elevated levels of 1,25(OH)$_2$D, despite very low levels of 25OHD. Second, serum levels of 1,25(OH)$_2$D don't necessarily tell anything about the intracellular levels of the hormone in muscle cells. Third, Geusens *et al.* [67] have shown clinical importance of VDR-genotypes, which support an *in vivo* effect of VDR-mediated effects. Thus, 1,25(OH)$_2$D seems to be involved in the pathogenesis of HDM. One hypothesis may reconcile the inconsistencies outlined above, namely the purported presence of intracellular, autocrine production of 1,25(OH)$_2$D from 25OHD in muscle cells [68]. There is an increasing amount of evidence suggesting the clinical importance of extrarenal 1,25(OH)$_2$D synthesis [101–107] (see Chapter 79). Two features distinguish extrarenal from renal synthesis of 1,25(OH)$_2$D: 1) it is not under control of PTH, but is dependent on the availability of the substrate 25OHD; 2) local 1,25-(OH)$_2$D synthesis has been shown to take place in the mitochondria [101]. Muscle has a very high content of mitochondria, which makes muscle a very likely site of extrarenal 1,25(OH)$_2$D synthesis. Intracellular production of 1,25(OH)$_2$D could explain the correlation between 25OHD and the muscular effects of vitamin D. Further, this pathway still requires 1,25(OH)$_2$D to be the final effector of vitamin D's muscular effects. It has been argued that local 1,25(OH)$_2$D synthesis in muscle should result in increased serum levels of 1,25(OH)$_2$D. Significant local 1,25OH$_2$D production has been identified in other tissues (endothelium [102], prostate [106], bone cells, liver cells, skin, etc. [103]), but these sites do not result in increased serum levels of 1,25(OH)$_2$D. The absence of increased 1,25(OH)$_2$D in these instances is most likely explained by the presence of highly-induced intracellular 24-hydroxylase

activity, ensuring degradation of $1,25OH_2D$ before it reaches the circulation. Also, failure of release of $1,25(OH)_2D$ into the circulation may be an additional factor.

C. 25OHD

25OHD is considered to be the storage and circulating form of vitamin D, and measurement of serum levels of 25OHD best reflect the vitamin D status of the body. 25OHD has been presumed to be biologically inert, but recent data challenge this notion. As already mentioned above, serum levels of 25OHD correlate to the biological effects of vitamin D *in vivo*. The effects of 25OHD on muscle cells could be mediated in several ways. 25OHD has some affinity for VDR, but the affinity of $1,25(OH)_2D$ for VDR is approximately 1000-fold higher than 25OHD. The serum concentration of 25OHD is about 500–1000 times higher than $1,25(OH)_2D$, but most is bound to vitamin D binding protein (DBP) (see Chapter 8). Competitive binding of the two vitamin D metabolites to VDR might be possible under some circumstances [108–110]. No specific receptor for 25OHD has been identified. As mentioned in the paragraph above, a more likely explanation is the local synthesis of $1,25(OH)_2D$ from 25OHD as substrate.

Finally, it is possible that 25OHD could exert direct effects on muscle via an effector-mechanism, which is still under investigation. Recently, Nykjaer *et al.* [111–113] (see Chapter 10) identified the cubilin-megalin receptor system as being responsible for renal reuptake of vitamin D metabolites bound to DBP. Muscle tissue possesses receptors of the LDL receptor family, which potentially could be involved in tissue specific uptake of 25OHD, but this still needs to be investigated (personal communication A. Nykjaer).

VIII. OTHER POSSIBLE MUSCULAR EFFECTS OF VITAMIN D

A. Insulin Resistance in Vitamin D Deficiency — Due To HDM?

Vitamin D deficiency has been reported to increase the risk of developing insulin resistance and abnormal oral glucose tolerance tests (OGTT) [114–117]. Striated muscle is central in the pathogenesis of Type 2 diabetes. GLUT4 is the most important glucose transporter in muscle [118–120]. The GLUT4 content of muscles declines with age [118,120], especially in the fast type II muscle fibers. Furthermore, GLUT4 is reduced in type 2 diabetes [119]. More research is necessary to establish a possible effect of vitamin D on the GLUT4 content of the muscle.

In type 2 diabetes, serum levels of free fatty acids are elevated [121,122]. Significant perturbations in the energy metabolism of mitochondria in muscle has been described in hypovitaminosis D [66], as well as in the presence of increased levels of PTH [97]. Additional research is warranted on the possible effects of vitamin D and PTH on fatty oxidation in striated muscle.

B. Possible Effects of Vitamin D on Muscle Regeneration

During exercise, serum levels of $1,25(OH)_2D$ have been reported to increase temporarily [123–127]. Exercise damages the muscle fibers and induces regeneration and growth of the muscle through enhanced satellite cell proliferation [128,129]. It could be speculated that $1,25OH_2D$ might be of importance in the regeneration process of muscle. Furthermore, reduced IGF-I levels seem to play a role in age-related muscle degeneration. A possible interrelationship between IGF-I levels and vitamin D levels should be investigated [130].

IX. SUMMARY

In this chapter we have reviewed the increasing evidence pointing to direct effects of vitamin D on striated muscle, making striated muscle an important target organ for vitamin D. Hypovitaminosis D myopathy (HDM) is a reversible disease that can recover completely, usually with significant improvement within a few weeks to a month after beginning vitamin D treatment [7,131,132]. Full restoration of severe HDM, however, may take 6 to 12 months of treatment with vitamin D [10]. Moreover, there is strong evidence for the prophylactic effects of vitamin D to reduce the risk of falls through improved muscular function and thereby to decrease the incidence of fractures. A daily dose of at least 800 IU (20 μg) cholecalciferol preferably in combination with 1000–1200 mg calcium seems to be the most effective treatment. Consequently combined vitamin D and calcium prophylaxis should be considered to combat hip fractures in the elderly. All patients at risk for vitamin D deficiency (i.e., lack of sunlight exposure) should be suspected to suffer from HDM. Those patients suspected of having HDM should have a blood test performed for measurement of 25OHD and PTH. In severe cases of HDM, treatment

should be initiated with a higher dose of vitamin D in order to speed up recovery. 300,000 IU cholecalciferol or ergocalciferol can be given either as an oral dose or intramuscular injection. This can be given as a single dose or repeated every month for three months. The high dose vitamin D should be combined with a daily supply of calcium.

In order to avoid HDM, the serum levels of 25OHD should be kept above 50 nmol/l and PTH levels should be suppressed to the normal range. Maintenance of normal 25OHD levels in the elderly should have a high priority, as hip fractures and disability carry a high cost for society as well as for the individual patients. Treatment of HDM results in significant improvement in quality of life. However, vitamin D is not the solution to every musculoskeletal problem in the aging population. The age-related loss of muscle power (approximately 1.5% per year [32]) seems to be obligatory and unrelated to vitamin D deficiency.

The data summarized in this review, lead to new questions, of which the ones, we consider most important are listed below:

1. Do muscle cells have the capacity to synthesize $1,25(OH)_2D$ from 25OHD?
2. Is hydroxylation of 25OHD to $1,25(OH)_2D$ necessary in order to mediate its effect on muscle, or does 25OHD have an effect of its own?
3. How do elevated PTH levels interact with vitamin D in muscle?
4. Finally, is the uptake of 25OHD and $1,25(OH)_2D$ in muscle a matter of simple diffusion, or do muscle cells possess a system for facilitated uptake of the compounds?

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CHAPTER 103

Renal Failure and Secondary Hyperparathyroidism

MASAFUMI FUKAGAWA Division of Nephrology & Dialysis Center, Kobe University School of Medicine, Kobe, Japan

KIYOSHI KUROKAWA Research Center for Advanced Science and Technology, The University of Tokyo, Tokyo, Japan

I. ROLE OF VITAMIN D IN THE DEVELOPMENT OF HYPERPARATHYROIDISM IN RENAL FAILURE

The kidney is the main organ for the production of active vitamin D, 1,25-dihydroxyvitamin D_3 ($1,25-(OH)_2D_3$) [1], a process that is catalyzed by 25-hydroxyvitamin D_3 1alpha-hydroxylase (1α-hydroxylase) in the proximal tubule cells [2–4]. Activity of this enzyme is attenuated in chronic renal failure due to phosphate load [5,6] as well as to the decreased numbers of viable nephrons [1]. Furthermore, it has recently been shown that fibroblast growth factor-23 (FGF-23) may suppress activation of vitamin D [7]. FGF-23 is a newly discovered phosphaturic factor (see Chapters 26 and 29) and increased serum levels have been reported in patients with renal dysfunction [8].

In addition to the decreased production of $1,25-(OH)_2D_3$ in the kidney, the importance of vitamin D deficiency has been recognized again, especially in chronic kidney disease (CKD) stages 3 and 4 [9]. Vitamin D deficiency is reflected by decreased serum concentrations of 25(OH)D [10]. Such decrease of 25(OH)D level may result from the loss of vitamin D-binding protein into the urine [11] as well as malnutrition [12]. In addition, a decrease of megalin on the brush border of proximal tubules has been reported [13], which results in diminished reuptake of filtered 25(OH)D [14] (see Chapter 10).

In chronic renal failure, the secretion of parathyroid hormone (PTH) is stimulated by several factors, primarily hypocalcemia and reduced production of $1,25(OH)_2D_3$ [1]. In addition, direct stimulatory action of phosphate on parathyroid has recently been demonstrated [15–17]. Thus, secondary hyperparathyroidism develops almost inevitably in patients with chronic renal failure without appropriate therapy [1]. Excess PTH accelerates bone turnover and results in a typical bone abnormality known as osteitis fibrosa [18].

Vitamin D metabolites suppress the secretion of PTH by correcting hypocalcemia and also by direct action on parathyroid cells in patients of chronic renal failure [19,20]. However, it is still difficult to suppress PTH secretion in substantial numbers of patients by vitamin D treatment. Such patients usually have marked parathyroid hyperplasia [21]. Since conventional uses of vitamin D in mild and advanced renal failure, including $1,25(OH)_2D_3$ pulse therapy, are discussed in the Chapter 76 by Dusso, Brown, and Slatopolsky, we will focus on patients with severe disease that are refractory to medical therapy and summarize the new therapeutic uses of vitamin D metabolites.

II. RESISTANCE TO $1,25(OH)_2D$ AS A CAUSE OF SEVERE SECONDARY HYPERPARATHYROIDISM IN CHRONIC RENAL FAILURE

A. Resistance to $1,25(OH)_2D$ in Chronic Renal Failure

Despite physiological plasma concentrations of $1,25(OH)_2D$, as well as those of calcium ion obtained by routine treatment, there are still many patients with elevated plasma PTH levels. Some of these patients respond to supraphysiological concentration of $1,25(OH)_2D_3$

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achieved either by intravenous or oral intermittent high doses of $1,25(OH)_2D_3$, which is also refered to as "$1,25(OH)_2D_3$ pulse therapy" [22–24]. These observations suggest that the resistance of parathyroid cells to $1,25(OH)_2D$ may play a major role in the pathogenesis of severe secondary hyperparathyroidism in chronic renal failure [25].

Resistance to physiological concentrations of $1,25(OH)_2D$ may develop during the early phase of chronic renal failure. In rat models of mild renal failure, PTH secretion, synthesis, and parathyroid cell proliferation were all enhanced even in the presence of a normal plasma concentration of calcium and $1,25(OH)_2D$ [26]. Hyperparathyroidism returned to normal with pharmacological doses of $1,25(OH)_2D_3$ without the induction of hypercalcemia. In these rats, $1,25(OH)_2D$ receptor (VDR) density in parathyroid glands, detected by Western blot, was decreased compared to levels seen in normal rats. Such reduction of VDR density in parathyroid glands also has been demonstrated in enlarged parathyroid glands of chronic dialysis patients [27], as well as in animal models of chronic uremia [28,29]. This abnormality, reduced VDR concentration, is currently considered the central feature responsible for the resistance of parathyroid glands to $1,25(OH)_2D$ in chronic renal failure [30].

In addition to the decreased density of VDR, several mechanisms have been proposed (Fig. 1). Decreased density of retinoid receptor X (RXR), which forms heterodimers with VDR, has been suspected [31]; however, the significance of this observation still remains unclear. Hsu and associates have been focused on the possible inhibition of $1,25(OH)_2D$ action by uremic toxins [32]. They have shown that serum from uremic patients inhibited the interaction between the $1,25(OH)_2D$-VDR complex and DNA [33], possibly through the formation of a Schiff base [33]. Although they have examined the effects of glyoxylate [35], other uremic toxins responsible for this inhibition still remain to be identified [36]. In addition, calreticulin has been shown to inhibit the binding of the $1,25(OH)_2D$-VDR complex to the vitamin D responsive element (VDRE) in the PTH gene promoter [37]. Hypocalcemia was found to induce increased concentrations of calreticulin exclusively in parathyroid glands. It is possible that this molecule plays some role in the regulation of VDR function by extracellular calcium [38,39]. Decreased action of $1,25(OH)_2D$ due to these mechanisms finally results in disturbed up-regulation of VDR, which further increases resistance to $1,25(OH)_2D$ in chronic renal failure (Fig. 1) [40–42]. Such a vicious cycle may be prevented by early vitamin D treatment as suggested by the animal models [43].

B. Advantages and Limitations of Intravenous Calcitriol Therapy

Considering that these abnormalities cause vitamin D resistance, it is quite reasonable that supraphysiological concentrations of $1,25(OH)_2D$ have been shown effective in suppressing PTH secretion in chronic dialysis patients resistant to conventional oral calcitriol [22–24]. Since the peak concentration of $1,25(OH)_2D$ is more important

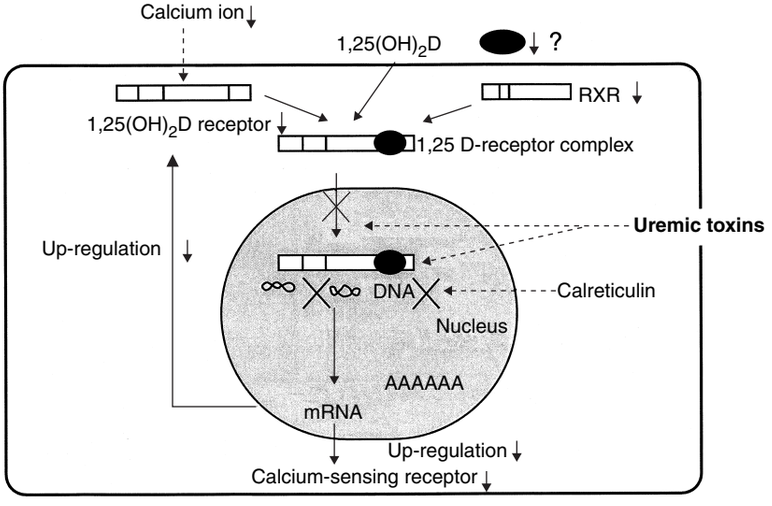

FIGURE 1 Mechanisms of resistance to $1,25(OH)_2D$ in chronic renal failure. Several steps of $1,25(OH)_2D$ action are disturbed in chronic renal failure, leading to further reduction of VDR.

for the suppression of PTH secretion than the total dose of calcitriol, as shown in dialysis patients [22] and in experimental animals [44], higher doses of calcitriol theoretically should be more effective. However, high doses of calcitriol often cause hypercalcemia and hyperphosphatemia, resulting in reduction or disconcentration of therapy. High Ca×Pi product leads to metastatic calcification including within blood vessels, which may result in a higher mortality risk [45]. This has been the main reason why less calcemic vitamin D analogs, such as 19-nor-1,25$(OH)_2D_2$ [46] and 22-oxa-1,25$(OH)_2D_3$ [47], have been developed (see Section VIII of this book). Even with these less calcemic vitamin D analogs, PTH secretion is still difficult to control in some patients.

C. Parathyroid Size as a Marker for the Prognosis of Vitamin D Therapy

In order to avoid unnecessary vitamin D treatment and metastatic calcification, it is certainly necessary to have a good predictor of the prognosis of vitamin D therapy for parathyroid suppression in these patients. Although recent data suggest that serum FGF-23 levels may be a marker for the future prognosis of hyperparathyroidism [48], parathyroid gland size assessed by ultrasonography is the most simple and useful marker at this time.

Marked parathyroid gland hyperplasia is a unique feature of secondary hyperparathyroidism in chronic dialysis patients [49]. Although the size of each of the four glands is usually different, even in the same patient, it has been recognized by experience that the size of the largest gland roughly correlates with the length and severity of uremia and with the degree of prevailing plasma and stimulated peak PTH levels [50,51]. The size also correlates with the degree of abnormal control of PTH secretion [52,53], which may be normalized by calcitriol pulse therapy [54].

Clinical observations of dialysis patients suggest that the size of the largest gland is the critical marker for the long-term prognosis of vitamin D therapy [55]. If the largest gland is larger than 1 cm in diameter or about 0.5 cm^3 in volume, it is usually difficult to suppress PTH secretion by calcitriol pulse therapy. In such patients, secondary hyperparathyroidism always persists or relapses even if it initially responded to calcitriol pulse therapy. By contrast, patients with only smaller glands usually respond well to calcitriol pulse therapy, and parathyroid gland function can be controlled then with oral active vitamin D sterols. Thus, the size of the parathyroid gland may have more relevance than plasma PTH levels in assessment of calcitriol pulse therapy [37]. Furthermore, Tominaga *et al*. demonstrated in patients treated by surgical parathyroidectomy that autoimplantation of tissue fragments from glands heavier than 0.5 g resulted in frequent relapse of hyperparathyroidism [56]. Thus, the critical size for the management strategy for hyperparathyroidism in chronic dialysis patients seems to be less than 0.5 cm^3 in volume.

The correlation between the gland size and the resistance to calcitriol can be explained by the degree of decrease of VDR density. VDR density is inversely correlated with the weight of enlarged glands [57]. Large parathyroid glands are usually composed of nodular hyperplasia, a more advanced type of pathology than diffuse hyperplasia seen in small glands [58]. It has been reported that cells in nodular hyperplasia glands have higher proliferative potentials [59,60,62] and more abnormal regulation of PTH secretion [63] than cells in diffuse hyperplasia glands. We and others have clearly shown that the VDR number was decreased more in nodular hyperplasia than in diffuse hyperplasia [57,64]. Since 90% of the glands heavier than 0.5 g were composed of nodular hyperplasia as shown by Tominaga and Takagi [61], the difference in the response to calcitriol that is dependent upon gland size can be explained by the difference in the type of hyperplasia in the larger glands.

In nodular hyperplasia, decreased density of the calcium-sensing receptor also has been demonstrated [65,66]. Although it is still controversial whether this decrease is the cause or the result of secondary hyperparathyroidism, a direct correlation between cell proliferation and decrease of calcium-sensing receptor has been suggested [67,68]. Thus, glands with nodular hyperplasia are less responsive to the suppressive effect of ambient calcium. This may partially explain the empirical finding of high PTH levels in the presence of hypercalcemia in patients with nodular hyperplasia. The progression of parathyroid hyperplasia is summarized in Fig. 2.

It is of note that some enlarged glands smaller than 0.5 cm^3 may be composed of nodular hyperplasia [61]. Nodule formation may be recognized by the shape of the glands detected by the latest models of ultrasonography devices [70]. Furthermore, Onoda *et al*. recently reported that positive blood supply detected inside the gland was highly suggestive of nodular hyperplasia [71].

III. MANAGEMENT OF SEVERE HYPERPARATHYROIDISM REFRACTORY TO MEDICAL THERAPY

Prevention of parathyroid hyperplasia from the early phase of chronic renal failure is the most important

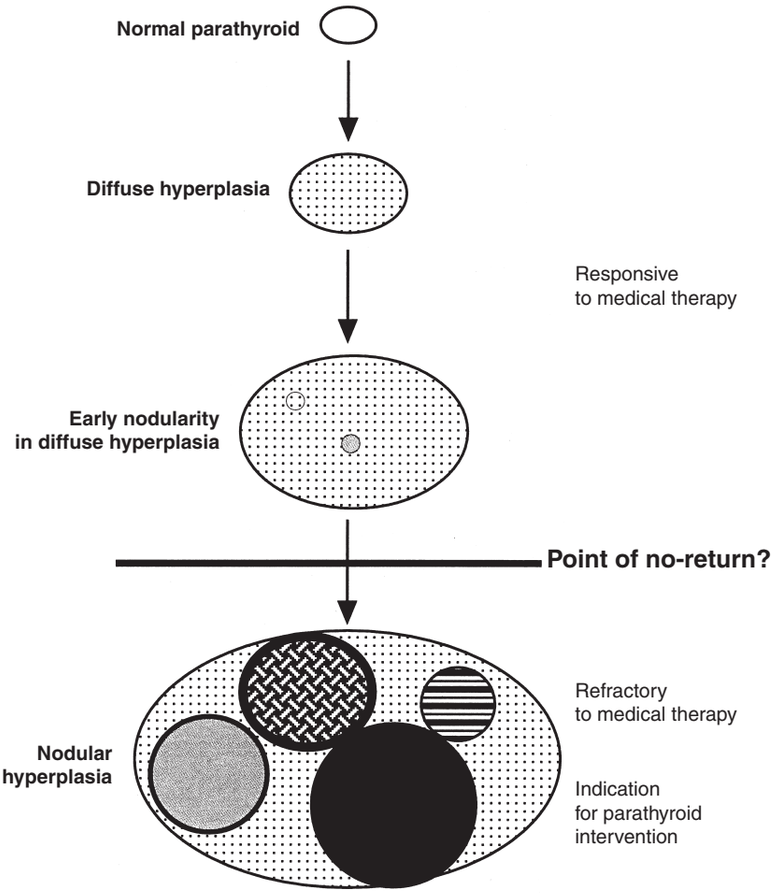

FIGURE 2 Progression of parathyroid hyperplasia in chronic renal failure. Cells with more severe reduction of VDR receptor and calcium-sensing receptor within diffuse hyperplasia form small nodules leading to nodular hyperplasia.

strategy for the management of secondary hyperparathyroidism in chronic renal failure. This can be achieved by dietary phosphate restriction and the early use of phosphate binders and cautious use of active vitamin D sterols as described in Chapter 76. Recent data suggest that the direct effects of phosphate on parathyroid cells is especially important in the early phase of chronic renal failure [72].

A. Selective Percutaneous Ethanol Injection Therapy (PEIT)

Although the introduction of new vitamin D analogs and calcimimetics may be promising in the treatment of secondary hyperparathyroidism, what can be done for patients with nodular hyperplasia? Do they have any choice other than surgical parathyroidectomy [73,74]. The ongoing discussion indicates that small glands composed of diffuse hyperplasia should still be responsive to calcitriol even in such patients. However, what about the patients that have already progressed to nodular hyperplasia?

For patients with nodular hyperplasia, two new techniques have been established. The first technique is the selective percutaneous ethanol injection therapy (PEIT) [75–77]. The second technique is direct vitamin D injection therapy (see below).

In PEIT, glands with nodular hyperplasia are "selectively" destroyed by ethanol injection under ultrasonographic guidance. Other glands with diffuse hyperplasia are then controlled by medical therapy (Fig. 3). Recently, this technique has become more powerful and safer than ever and has become widely used, especially in Japan. According to the guideline by Japanese Society for Parathyroid Intervention [78],

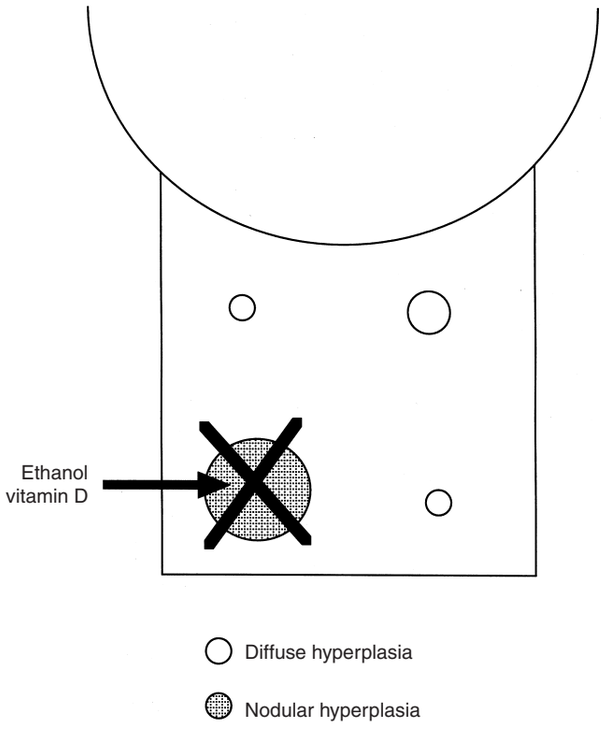

FIGURE 3 Parathyroid intervention under ultrasonographic guidance. Glands with nodular hyperplasia (hatched circle) are treated by intervention using percutaneous ethanol injection therapy (PEIT). Other glands with diffuse hyperplasia (open circle) are then controlled by medical therapy.

patients having one or two glands with nodular hyperplasia are the best candidates for PEIT. After the initial ethanol injection, PTH levels and recurrence should be monitored carefully. More importantly, after the successful destruction of nodular hyperplasia, residual glands with diffuse hyperplasia should be managed by appropriate medical therapy, including dietary phosphorus restriction. Thus, good compliance of the patients to medical therapy and regular check-ups after PEIT is essential.

In the largest study by Kakuta *et al.*, 46 patients were treated by selective PEIT on an outpatient basis followed by appropriate medical therapy. PTH levels in 80% of the patients remained within the target range at one year after initial treatment [79]. Long-term follow-up (three years) after PEIT has been also reported by this group [80].

Failure of PTH suppression despite successful ablation of glands with nodular hyperplasia suggests the existence of another gland containing nodular hyperplasia beyond the reach of ultrasonography [81]. If ectopic glands are recognized before the procedure is performed, initial surgical parathyroidectomy is indicated. Thus, it may be reasonable to search for ectopic glands before PEIT in patients with more than three glands of critical size.

Recurrent nerve palsy due to leakage of ehanol is one of the most important complications of PEIT [77]. It is also suspected that adhesions in the tissue surrounding the parathyroids may be caused by leakage of ethanol. This could be a major problem if surgical parathyroidectomy will be needed in the future. With the routine use of color Doppler flow mapping by ultrasonography, the volume of ethanol used for PEIT has become minimal, leading to the lower rate of such complications [79]. Nevertheless, skilled operators and appropriate equipments are certainly required for successful and safe PEIT [78].

B. Direct Vitamin D Injection Therapy

The second technique for treating nodular hyperplasia is direct calcitriol injection therapy under ultrasonographic guidance (PCIT) [82]. In this therapy, very high local concentration of $1,25(OH)_2D_3$ or other vitamin D metabolites is achieved exclusively in injected parathyroid glands. As already reported, direct calcitriol injection therapy not only suppressed PTH secretion, but also restored the responsiveness to medical therapy. Such effects have been confirmed by other studies with different protocols [83–85]. Furthermore, direct injections of 22-oxacalcitriol have also been tried with favorable results [86].

In contrast to PEIT, the risk of recurrent nerve palsy is extremely low with PCIT. However, a recent report suggested that direct injection of 22-oxacalcitriol might evoke inflammation, resulting in adhesions of the surrounding tissue [87]. The use of vitamin D analogs is promising and such risks should be avoidable with improvements in technique. Future development may allow direct injection of new calcimimetics and even adenovirus-mediated gene transfer to treat parathyroid hyperplasia, as has been shown recently in animal models [88].

C. Regression of Parathyroid Hyperplasia: Is It Really Possible?

The cell cycle of parathyroid cells is usually very slow, even in the hyperplastic glands [89]. Although prevention of parathyroid hyperplasia by several therapeutic modalities has been demonstrated in rat models of chronic kidney disease [90], suppression of PTH secretion may not lead to the complete normalization of parathyroid cell function including proliferation site in patients with secondary hyperparathyroidism [91,92].

Thus, it is still an unsettled issue whether hyperplastic parathyroid glands do regress after proper medical therapy or after kidney transplantation.

Regression of parathyroid hyperplasia has been reported in chronic dialysis patients treated by oral calcitriol pulse therapy [93–95], although controversial data have been also reported [96]. Such a regression was observed not only in cases with successful PTH suppression, but also in small glands even in cases without significant suppression of PTH [55,97]. Thus, in our opinion it is reasonable to conclude that glands with diffuse hyperplasia regress after effective medical therapy. In contrast, as discussed above, glands with nodular hyperplasia do not regress except for a few cases in which spontaneous apoplexy of the gland was suspected [98,99].

Due to the lack of established parathyroid cell lines for *in vitro* studies, mechanisms of regression have not yet been satisfactorily elucidated [100]. In order to achieve regression of hyperplastic glands, suppression of cell proliferation may not be sufficient. Negative cell balance by increased apoptosis may be needed. However, in rats, it has been very hard to demonstrate the apoptosis of parathyroid cells, which takes place in a limited number of cells during cell turnover [101–104]. Moreover, interpretation of apoptotic cells demonstrated in surgically removed parathyroid glands in dialysis patients has been controversial [100].

In a 1977 report by Henry *et al*. [105], reduction of parathyroid cell number was clearly demonstrated in three-month-old vitamin D–deficient chickens treated with vitamin D replacement. In contrast, $1,25(OH)_2D_3$ treatment suppressed parathyroid cell proliferation, but did not reverse hyperplasia in experimental uremia, as demonstrated by Szabo *et al*. [106]. In recent animal studies by Lewin *et al*. [107,108], hyperparathyroidism induced by long-term uremia returned to normal following kidney transplantation. However, parathyroid hyperplasia was persistent. Such a suppression of PTH secretion with persistent hyperplasia has also been demonstrated in rat models of secondary hyperparathyroidism induced by high phosphorus diet, by switching to low phosphorus diet. In these animal models, reversal of reduced VDR or calcium-sensing receptor has not been confirmed at least in the short term.

As discussed above, it has been suggested recently that regression of nodular hyperplasia may be induced by direct vitamin D injection therapy, originally performed with calcitriol [82]. By injecting directly into enlarged glands under ultrasonography, very high local concentration of vitamin D or analog can be achieved transiently. Shiizaki *et al*. recently reported that direct injection of 22-oxa-calcitriol solution into enlarged glands in patients leads to the regression of hyperplasia [84]. By repeated parathyroid biopsy before and after the therapy, they clearly demonstrated the induction of apoptosis of parathyroid cells in the injected glands. They also suggested that such a regression was associated with up-regulation of VDR and the calcium-sensing receptor. These data suggest that direct vitamin D injection therapy not only induces apoptosis of parathyroid cells, but also restores the responsiveness of residual parathyroid cells to medical therapy, leading to normalization of parathyroid hyperplasia. It may be possible that such specific effects of vitamin D on parathyroid cells may also be achieved by oral or intravenous preparations, if vitamin D analogs with these specific actions can be designed in the future.

It is also of note that increased 25-hydroxyvitamin D_3 1α-hydroxylase and reduced 25-hydroxyvitamin D_3 24-hydroxylase expression have been reported in parathyroid tumors [109]. Thus, parathyroid is not only a target organ of vitamin D, but it also metabolizes vitamin D. Since parathyroid glands possess 1α-hydroxylase, it may become possible to develop new vitamin D metabolites that use this system to be activated only in parathyroid.

IV. FUTURE ROLES OF VITAMIN D ANALOGS IN CHRONIC RENAL FAILURE

A. Design of Vitamin D Analogs with Specific Actions on Specific Tissues in Chronic Renal Failure

The parathyroid glands are not the only target organ of vitamin D therapy in patients with chronic renal failure. The skin and the immune system are other examples; however, the role of vitamin D treatment on these systems, as well as other organs, has not been fully clarified yet [110,111]. A recent report suggests that paricalcitol treatment leads to better survival than calcitriol treatment in chronic dialysis patients [112]. It is still unclear whether such a difference is due to the less calcemic effect of paricalcitol or to its effects on other organ systems.

Bone is a representative classic target organ of vitamin D. Thus, different effects of 22-oxacalcitriol versus calcitriol on bone turnover have been noted in animal models of chronic renal failure [113] (see Chapter 86). As recently suggested, the different effects of vitamin D analogs on bone, seen in *in vitro* and *in vivo* experiments, might be clues that help in the future elucidation of the mechanism for the differential actions of analogs in various tissues [114]. 22-oxacalcitriol was originally developed as an analog

with potent activity on the differentiation of leukemic cell lines [115]. Such an activity has also been examined with paricalcitol [116]. Recently, it has been demonstrated that different vitamin D analogs utilize specific cofactors for target gene regulation [117]. Different cofactors bind to different genes, evoking different actions on the same cell. Thus, it is expected that design of vitamin D analogs with differential actions in specific organs will become possible [118]. For example, it may be possible to design analogs that specifically induce apoptosis of parathyroid cells as well as less calcemic analogs.

B. Possible Treatment of Chronic Kidney Disease by Vitamin D Analogs

The kidney is not only the site of active vitamin D production, but also is its target organ. As intensively discussed in several previous chapters, vitamin D metabolites modulate the activity of the enzymes involved in vitamin D synthesis and degradation. Calcium-binding proteins [119] are also induced by vitamin D in the distal tubules of the kidney. VDRs have been identified in various parts of the kidney and no doubt are regulated by vitamin D metabolites [120]. Inhibition of renal cell proliferation by vitamin D was initially demonstrated in renal cell carcinoma lines [121]. It has also been shown that $1,25(OH)_2D_3$ diminished ^{3}H-thymidine incorporation, cell counts, and TGF-β secretion into the supernatant of cultured proximal tubular cell lines [122–124] and in cultured human mesangial cells [125]. Regulation of mesangial cell smooth muscle phenotype has also been suggested [126].

In vivo, $1,25(OH)_2D_3$ reduced renal weight, protein content, DNA content, and the number of mitoses in the remnant kidney with compensatory hypertrophy after uninephrectomy [127]. On the contrary, $1,25(OH)_2D_3$ may induce type IV collagen synthesis, possibly through up-regulation of TGF-β type II receptor [126] and up-regulation of protein-1 [128].

It may be possible that vitamin D ameliorates glomerular injury seen in chronic kidney disease, although the effects may depend on the phase of renal injury. It has recently been shown that $1,25(OH)_2D_3$ inhibited progressive glomerulosclerosis in subtotally nephrectomized rats [129] and reduced proteinuria, glomerular hyper-cellularity and inflammatory infiltration in anti-Thy-1.1 nephritis [130]. Similar suppressive effects have been also demonstrated with retinoic acids [131,132].

These studies suggest the possibility that vitamin D may alter the rate of progression of CKD. In contrast, there has been a concern that oral vitamin D treatment in CKD patients may increase the risk of accelerating the progression of renal dysfunction by increasing urinary calcium excretion. In this respect, the less-calcemic vitamin D analogs may be more suitable for this purpose [133].

22-oxa-calcitriol is one such less-calcemic vitamin D compound used for the treatment of severe secondary hyperparathyroidism in chronic dialysis patients [134], as extensively reviewed in Chapter 86. It has recently been shown that 22-oxa-calcitriol also effectively ameliorated glomerular sclerosis in two rat models of chronic kidney disease without affecting calcium and phosphorus levels [135,136]. Although further studies are needed, a vitamin D analog with such properties may be a promising agent for the treatment of chronic kidney disease in the near future.

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CHAPTER 104

Inhibition of Benign Prostatic Hyperplasia by Vitamin D Receptor Ligands

MARIO MAGGI Andrology Unit, Department of Clinical Physiopathology, University of Florence, Florence, Italy

CLARA CRESCIOLI Endocrinology Unit, Department of Clinical Physiopathology, University of Florence, Florence, Italy

LUCIANO ADORINI BioXell, Milan, Italy

I. INTRODUCTION

The prostate is a gland surrounding the male urethra below the neck of the bladder and producing the prostatic fluid, a secretion which contributes 30% to the total ejaculate. The prostatic fluid is rich in fibrinolytic enzymes, such as prostatic-specific antigen (PSA), acid phosphatase, citric acid, and zinc. In humans, the prostate gland is composed of 40 to 50 ducts distributed essentially in three distinct zones: peripheral, central, and transitional or periurethral. While cell transformation in the peripheral zone gives rise to prostate cancer, cell growth in the periurethral zone leads to the most common age-related disease of the male: benign prostatic hyperplasia (BPH). The prostate weight is only a few grams at birth, and it increases during puberty, reaching approximately 20 g in the young adult. In contrast to the pubertal growth phase, which involves the entire gland, during the fifth decade of life, in the majority of men, there is a second growth phase selectively involving the periurethral zone leading to BPH [1]. The prevalence of BPH increases with age so that by age 80, about 90% of men have histological evidence of BPH [1]. In a subset of elderly men (27–35%), BPH can cause lower urinary tract symptoms (LUTS), which may require medical or surgical treatment [2] due to the compression by the enlarged prostate of the prostatic urethra, which decreases bladder outflow. In the earliest stages, this obstruction is compensated by an increased activity of the bladder detrusor muscular system but eventually complete voiding of the bladder is prevented, due to the slackening of the neck musculature. Urinary obstruction and even renal insufficiency might follow. This may lead to emergency surgery for acute urinary retention with an increased risk of morbidity and even death when compared to elective surgery [3].

II. PATHOGENESIS OF BPH

Periurethral prostate overgrowth involves both epithelial and stromal components, including both fibroblasts and smooth muscle cells, in various combinations. One of the earliest events in BPH development is the reduction of the epithelium/stroma ratio, most probably due to an imbalance between growth and death programs. Indeed, in the hyperplastic prostate, the epithelium regularly undergoes apoptosis, whereas stromal cells escape it [4], with a consequent increase in the stromal volume [5]. In addition, stromal growth factors (GFs) induce epithelial overgrowth and glandular hyperplasia [6,7]. All these events are clearly androgen-dependent, as shown by the observation that BPH does not develop in hypogonadal men, and that either surgical or pharmacological castration results in a decrease gland size [8–10]. However, BPH develops mainly in older men, when circulating testosterone, and in particular free testosterone, is progressively decreasing. Therefore, it is possible that sensitivity to androgens, rather than circulating androgen levels, are involved in BPH pathogenesis. A higher transcriptional activity of the androgen receptor (AR) due to a decreased number of CAG repeats in exon 1 has been reported in the majority [11–13], although not in all studies [14,15]. Interestingly, in hypogonadal

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patients the effect of androgen substitution on prostate growth was inversely related to the extent of CAG residues [16]. In addition, a recent study has indicated a decreased expression of the AR co-repressor DAX-1 in BPH [17]. These studies support the view that AR activity is up-regulated in the prostate of BPH patients. Therefore, blocking AR activity represents a promising approach in the treatment of BPH. This could be achieved by reducing androgen levels, for example, blocking their formation with GnRH analogs or by antagonizing androgen activity at the receptor level using AR antagonists. Although both these strategies might be indeed effective, in clinical practice they are unacceptable because of the major side effects caused by complete androgen ablation in otherwise healthy individuals. Wilson 1972 [18], first hypothesized that the main androgen inducing prostate hyperplasia was not testosterone (T), but its highly biologically active metabolite dihydrotestosterone (DHT), which is formed locally by two 5α-reducing iso-enzymes (5α-reductase type 1 and 2, the latter being predominant, see [19] for review). Interestingly, intra-prostatic DHT content is not decreased as a function of age [20–22]. According to Wilson's original hypothesis, blocking DHT formation with a type 2 selective (finasteride) or with a dual (dutasteride) inhibitor of 5α-reductase isoforms is, indeed, an effective treatment for BPH [23,24]. However, prostate size reduction obtained with this strategy is relatively limited (about 25%). Also some men experience sexual side effects related to partial androgen deficiency (decreased libido and impotence) that are not well tolerated, in particular in the ageing male [3,25]. It is possible that the limited clinical response to 5α-reductase inhibitors is due to a compensatory increase in intra-prostatic growth factor (GF) receptors, which follows androgen deprivation [26,27]. Therefore, an alternative strategy to reduce age-related prostate overgrowth is to decrease the activity of androgen-induced prostatic GFs, which are considered to mediate, at least partially, the proliferative activity of sex steroids in the gland [7,28,29]. It is interesting to note that the prostate gland is one of the few androgen targets retaining a proliferative responsiveness to androgens in adulthood. Therefore disrupting androgen-induced, intra-prostatic GF signaling is an attractive option to obtain a selective, and sexual side-effect free, therapy for BPH.

III. EFFECTS OF ANDROGENS AND GROWTH FACTORS ON HUMAN BPH CELLS

In the human prostate, the AR is expressed in both the epithelial and the stromal compartments and regulates mutual interactions between the two compartments (reviewed in [30]). However, as discussed above, stromal rather than epithelial cells are thought to be primarily involved in the pathogenesis of BPH. As shown in Fig. 1, BPH-derived stromal cells (BPH cells) express the AR gene and protein, with a high affinity for the ligand ($K_d = 72 \pm 34$ pM), as well as both isoforms of 5α-reductase [31]. In addition, they respond to androgens with an increased growth ($EC_{50} = 380 \pm 200$ pM, [31]). An increase in BPH cell proliferation was also obtained with addition of specific GFs, such as epidermal growth factor (EGF [32]), keratinocyte-GF (KGF [32,33]), and insulin-like growth factor-I (IGF-I [34]). Data in Fig. 2 show the maximal stimulatory activity of KGF (10 ng/ml), Des (1–3) IGF-I (an IGF-I analog which does not bind to binding proteins, 10 ng/ml) and T (10 nM) on BPH cell proliferation. KGF- and Des [1–3] IGF-I-induced proliferation was completely blocked only by specific antibodies, but not by unrelated antibodies or immunoglobulins (Fig. 2). Conversely, testosterone-induced cell growth was completely abolished not only by an AR antagonist (cyproterone acetate) or by a type 2 5α-reductase inhibitor (finasteride), but also by antibodies against the receptors for KGF (KGFR) and IGF-I (IGFR1) (Fig. 2). This indicates that T-induced proliferative activity in BPH cells is at least partially mediated by KGFR and IGFR1. This finding is consistent with data from organ cultures of neonatal rat ventral prostates, in which exogenous administration of KGF completely replaces the requirement of T for prostate growth and branching morphogenesis [35]. In addition, KGF has been also shown to replace androgen in eliciting growth and differentiation of seminal vesicles [36]. Hence, KGF, the predominant fibroblast GF (FGF) in human prostate [37], is considered one of the main prostatic andromedins, that is mediators of androgen-induced growth [38]. Also, the IGF system has been implicated in the pathogenesis of BPH. IGFR1 and IGF-II expression were higher in the periurethral zone of the human prostate than in other zones, and IGF-II levels were strictly correlated with the intra-prostatic androgen level [39]. Patients with the highest circulating levels of IGF-I have an elevated risk of BPH [40] and transgenic mice overexpressing IGF-I protein in the prostate show sign of hyperplasia in the ventral lobe, the most androgen-dependent zone [41].

IV. VITAMIN D RECEPTOR EXPRESSION IN PROSTATE CELLS

The aforementioned experimental and clinical studies indicate that an ideal medical treatment for BPH might be an agent able to disrupt the intra-prostatic

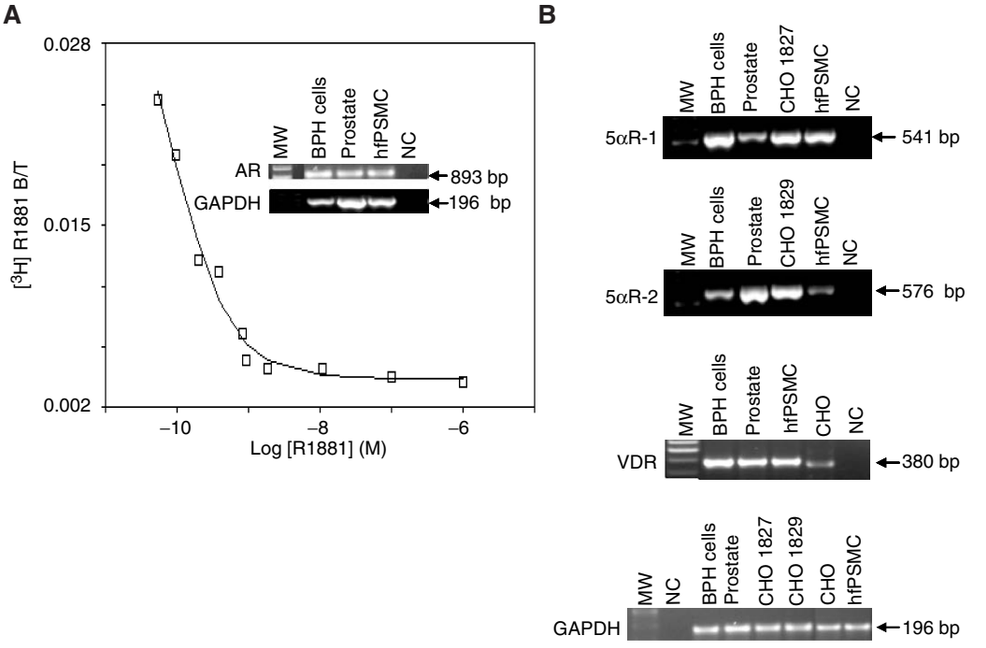

FIGURE 1 *Expression of AR, 5α-reductase and VDR in human BPH cells*. **Panel A**: homologous competition curve for [^{3}H]R1881 binding. R1881 binds with high affinity (K_d = 72 ± 34 pM) and low capacity (B_{max} = 2.64 ± 0.5 fM) to a single class of sites. *Ordinate*: B/T, Bound to total ratio for [^{3}H]R1881; *Abscissa*: Total concentration (molar) of labeled and unlabeled R1881. Inset: RT-PCR detection of the AR gene. Products are derived from total RNA using specific primers for AR (upper panel) and GAPDH (lower panel). **Panel B**: RT-PCR amplimers from total RNA of BPH cells, prostate tissue, CHO 1827 (transfected with 5α-reductase 1, 5α-R1, gene) or CHO 1829 (transfected with 5α-reductase 2, 5α-R2 gene), CHO cells, human fetal penile smooth muscle cells (hfPSMC), using specific primers for 5α-R1 (upper panel), 5α-R2 (second panel), VDR (third panel) and GAPDH (bottom panel). CHO 1827 or 1829, hfPSMC and human prostate were used as positive controls for 5α-R1, 5α-R2 and AR. GAPDH mRNA amplification was performed to verify the integrity and loading of the extracted total RNA. MW, molecular weight markers; NC, negative control. The blots are representative of three separate experiments.

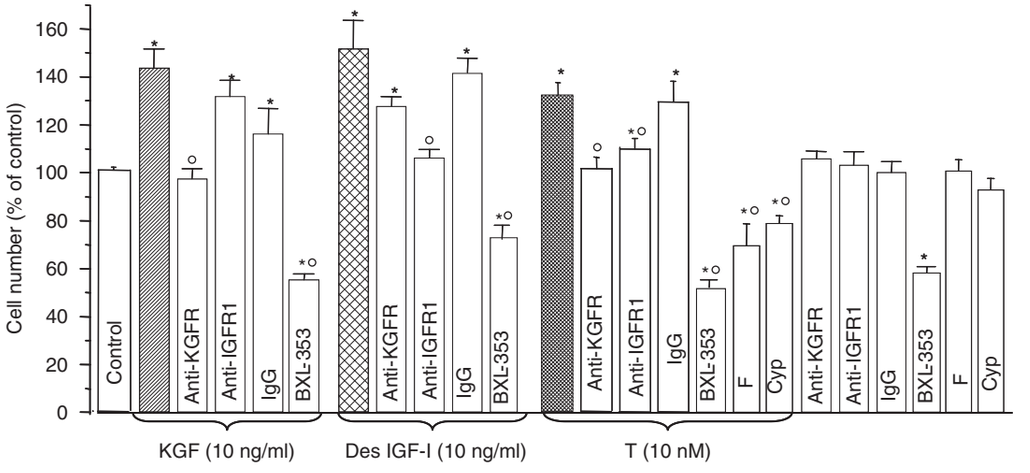

FIGURE 2 *Antiproliferative effect of BXL-353 on BPH cell growth induced by GFs or testosterone (T).* Incubation for 48 h with KGF (10 ng/ml), Des [1–3] IGF-I (10 ng/ml) or T (10 nM) significantly induced BPH cell proliferation. Anti-androgens such as the 5α-R2 inhibitor, finasteride (F, 1 nM), or the AR antagonist cyproterone acetate (Cyp, 100 nM) completely reverted T-induced stimulation of BPH cell growth, but they did not exert any effect on basal cell growth. Specific antibodies against KGFR (Anti-KGFR, 1 μg/ml) and IGFR1 (Anti-IGFR1, 1 μg/ml) blocked cell growth stimulated by their cognate GFs. T-induced cell proliferation was blunted by both types of anti-GF receptor antibodies. Immunoglobulin controls (IgG 1 μg/ml) failed to block either GF- or T-stimulated proliferation. BXL-353 (10 nM) was able to block BPH cell growth both in basal condition and in the presence of T or GFs. Results are expressed as percent increase (mean ± SEM) over their relative controls in 4 different experiments performed in quadruplicate (* $P < 0.01$ vs control; ° $P < 0.01$ vs GF- or T- treated cells by one-way ANOVA and paired or unpaired Student's t tests). The data are derived from Crescioli *et al*., 2003.

cross-talk between AR and GFs but devoid of anti-androgenic properties. Calcitriol analogs might comply with such criteria. The strict inter-relationships between vitamin D and prostate have been extensively described. Vitamin D deficiency has been proposed to be a risk factor for prostate cancer [42,43], because prostate cancer mortality in the USA increases as the availability of ultraviolet light exposure, and therefore of vitamin D formation, decreases [44] (see Chapter 90). Polymorphisms in the VDR gene have also been associated with increased risk of prostate cancer in some studies [45–47] (see Chapter 68). Malignant prostate cells express the VDR, and treatments with calcitriol, or less-hypercalcemic analogs, can inhibit prostate cancer proliferation and invasiveness (see Chapter 94 and ref. [48,49] for review). Interestingly, also epithelial and stromal cells of both human [50,51] (see also Figs. 1 and 2) and rat [51] normal prostate cells express the VDR, and addition of 1,25 $(OH)_2D_3$ inhibits cell growth [50].

V. ANTIPROLIFERATIVE EFFECTS OF BXL-353 ON HUMAN BPH CELLS

We have extensively studied the antiproliferative effects of VDR ligands, and in particular of 1,25-dihydroxy-16-ene-23-yne D_3 (BXL-353 or analog V), a compound 30-fold less calcemic than calcitriol, on human stromal prostate cells (BPH cells). BPH cells were obtained from prostate tissues derived from patients, who underwent suprapubic adenomectomy for BPH and did not receive any pharmacological treatment in the three months preceding surgery [33]. BPH cells showed positive staining for smooth-muscle actin, vimentin, and desmin, suggesting fibromuscular morphological features. Conversely, they were negative for epithelial and endothelial markers such as cytokeratin and factor VIII [33]. As shown in Fig. 2 BXL-353 completely inhibited GF- or T-induced BPH cell proliferation and also decreased the growth of unstimulated cells [31,33,34]. To better understand the antiproliferative effect of BXL-353, we have studied its effects on the cell cycle distribution of partially synchronized BPH cells after a 24 h culture with medium or KGF (10 ng/ml). As shown in Fig. 3, after serum starvation, more than 75% of the cells were in G_0/G_1-early S phase, as indicated by fluorescence emission of propidium iodide-stained nuclei. Treatment with KGF allowed the cells to progress through the cell cycle with a statistically significant decrease in the proportion of cells accumulated in G_0/G_1-early S and an increase in cells traversing the G_2/M phase. The simultaneous addition of BXL-353 completely antagonized the KGF-induced effects on cell-cycle progression.

In BPH cells GFs and steroids not only stimulated DNA synthesis and cell proliferation but also prolonged cell survival, via induction of the anti-apoptotic protein Bcl-2 [31,33,34] (see also Fig. 4). Members of the Bcl-2 family are essential mediators of cell survival and apoptosis, and include both anti- and pro-apoptotic intracellular proteins residing at the mitochondrial outer membrane [52–55]. Their classification is based on the presence or absence of Bcl-2 homology (BH) domains: BH1, BH2, BH3, and BH4 [56]. In particular, Bcl-2 and Bcl-XL members, both containing all four BH domains, inhibit apoptosis and promote cell survival [57]. Bcl-2 activity, derived by integrating signals from survival and death stimuli, seems to be regulated by several different mechanisms, like homo- and heterodimerization with other family members, or post-translational modifications such as phosphorylation and proteolysis [52,58]. BXL-353 not only dramatically reduced GF- or T-induced Bcl-2 overexpression and survival, but also in the presence of these anti-apoptotic factors was able to stimulate a sustained death program (Fig. 4). Hence, in BPH cells, BXL-353 induced a decrease in the progression through the cell cycle and an increase in the rate of programmed cell death. Similar results were observed with calcitriol in breast cancer cells [59], in the androgen-dependent prostate cancer cell line LNCaP [60,61] as well as in metastatic Dunning rat prostate carcinoma [62]. Interestingly, in LNCaP cells, overexpression of Bcl-2 completely blocked calcitriol-induced apoptosis but only partially affected cell cycle arrest [61], indicating that partially independent pathways mediate the effects of calcitriol on cell proliferation and cell death.

The inhibitory effect of BXL-353 on prostate growth and survival is at least partially explained by the inhibition of GF-induced receptor activation. We have found that a rapid incubation of both benign [33] and malignant [63] prostate cells with BXL-353 dramatically reduces agonist-induced KGF-R auto-phosphorylation, one of the earliest event of KGF signaling. Because this effect was rapid, induced in a few minutes, and accompanied by an increase in intracellular calcium concentrations [33], we speculated the involvement of a nontranscriptional mechanism which, in agreement with recent results in chick myoblast [64], might involve the same VDR translocated from the nucleus to the microsomal fraction. Other studies have shown that rapid effects of calcitriol are mediated by a binding protein different from the VDR [65–68].

It has also been shown [69] that the VDR, upon ligand binding, physically interacts with the catalytic subunit of protein phosphatases PP1 and PP2Ac, thereby promoting their enzymatic activities with the consequent inactivation of $p70^{S6k}$, a kinase essential

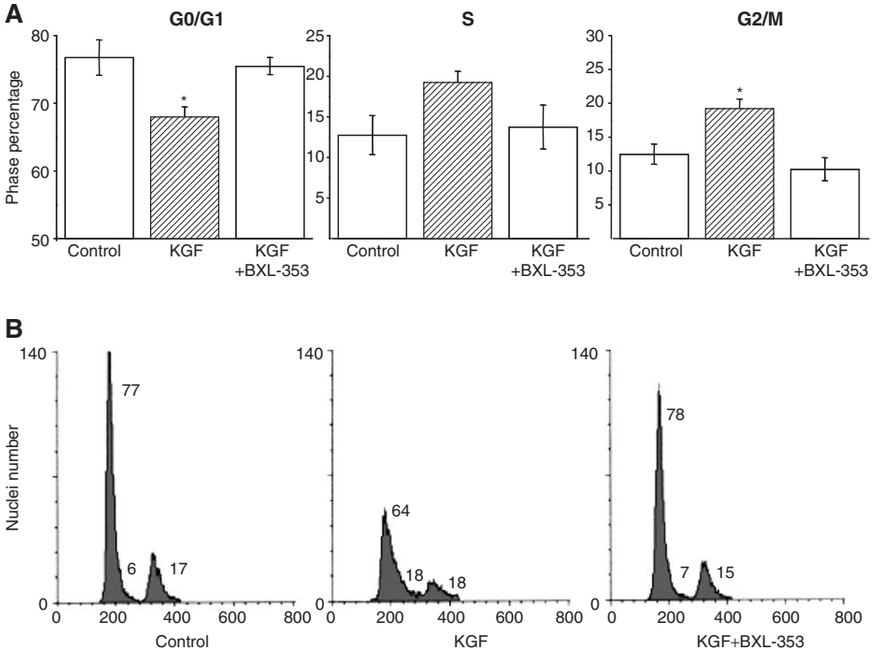

FIGURE 3 *Effect of BXL-353 on cell cycle distribution of BPH cells.* **Panel A** shows the effect of 24 h treatment with KGF (10 ng/ml) with or without BXL-353 (10 nM) on partially synchronized (24 h serum starvation) BPH cells. KGF significantly reduced the number of BPH cell accumulated in the G_0/G_1 phase, thereby increasing the percentage of nuclei in G_2/M. Simultaneous treatment with BXL-353 completely blocked the mitogenic activity of KGF. Results are expressed as cell cycle phase percentage. * $P < 0.01$ vs. control by one-way ANOVA and paired or unpaired Student's t tests. For assay method see ref. [86]. **Panel B** shows results from a typical experiment.

in G_0/G_1 transition. VDR ligands not only induced a prompt decrease in phosphorylated KGFR [33,63], but also in phospho-Erk and phospho-Akt [70,71]. Hence, it is possible that calcitriol and related analogs might activate the catalytic subunit of distinct families of phosphatases, leading to antiproliferative effects by targeting GF signaling. Interestingly, in the human epidermoid A431 cells, overexpressing an autocrine growth loop for EGF, calcitriol not only induced a rapid alteration of EGFR auto-phosphorylation (as previously observed by us on KGFR), but also impaired EGFR membrane trafficking and signaling via the classic VDR-dependent mechanism [72]. In conclusion, it is possible that genomic (nuclear VDR-dependent) as well as rapid or nongenomic (cytoplasmic VDR-dependent?) mechanisms simultaneously contribute to the growth-suppressing activity of VDR ligands on prostate cells.

VI. INHIBITION OF *IN VIVO* PROSTATE GROWTH BY BXL-353

To investigate whether calcitriol analogs might represent a new opportunity to decrease prostrate cell overgrowth and, therefore, to treat BPH, we carried out a series of studies using the rat as an experimental model. Taking advantage of the high sensitivity to androgens of the ventral prostate, castrated rats were supplemented with T with or without increasing doses of BXL-353 for various time periods [31]. Changes in ventral prostate volume and morphology, along with measurements of calcemia and hormonal values were studied. BXL-353 has a maximum tolerated dose of 30 μg/Kg, and at any dose tested never caused hypercalcemia. One week treatment with BXL-353 was sufficient to decrease significantly and dose-dependently ventral prostate weight, with an $IC_{50} = 1.5 \pm 1$ μg/Kg (Fig. 5, panel A). Similar results were obtained with a two-week treatment of BXL-353 (Fig. 5, panel B). A 30% reduction of ventral prostate weight was induced by one month treatment with BXL-353 to intact adult rats (Fig. 5, panel C). It is interesting to note that prostate weight reduction induced by BXL-353 (~50% decrease) is similar to that obtained in similar experimental models with 10 mg/Kg finasteride [31].

Because we observed that human BPH stromal cells underwent apoptosis even after short-term *in vitro* exposure to BXL-353, we investigated the fate of rat

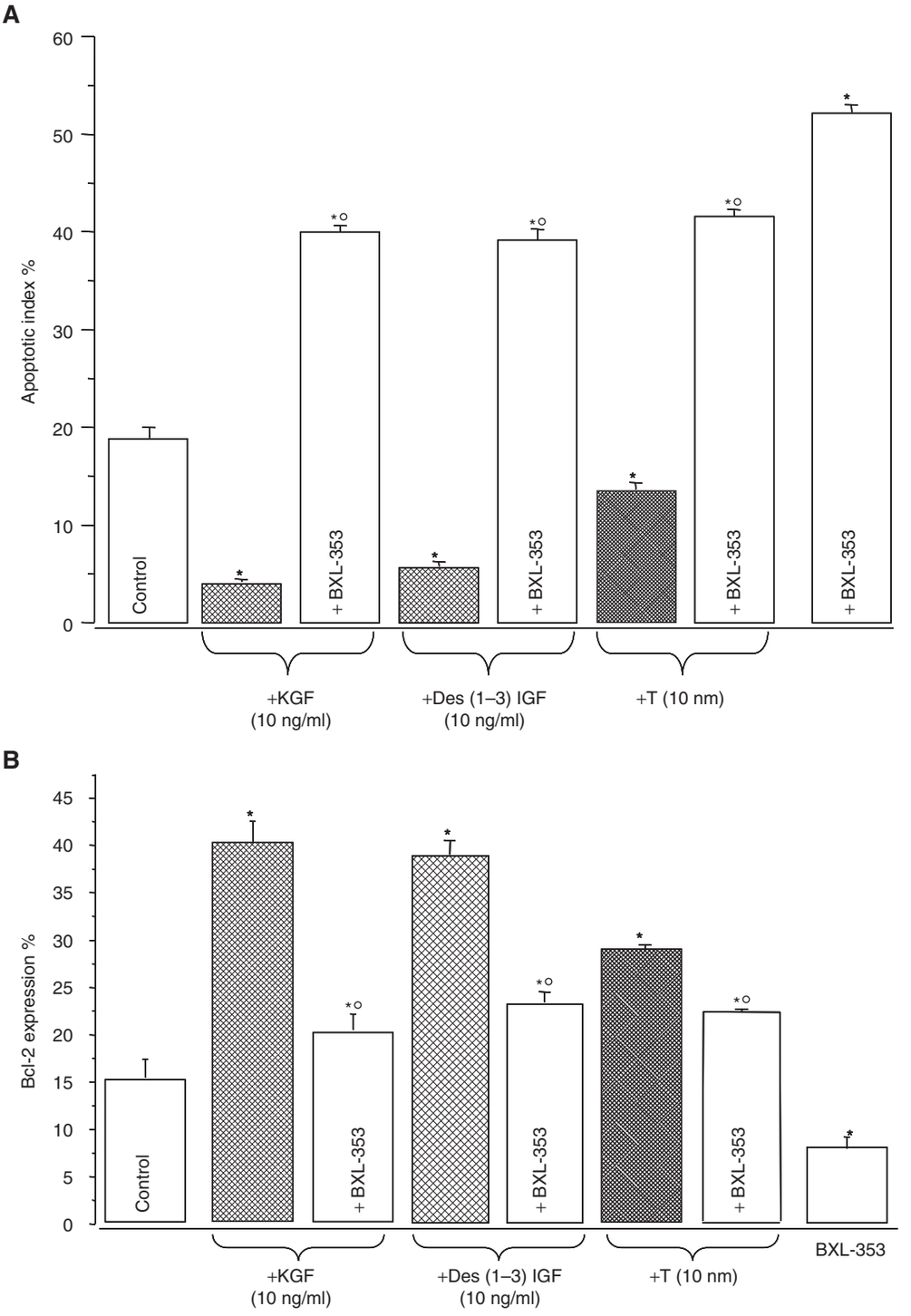

FIGURE 4 *Effect of BXL-353 on apoptosis and Bcl-2 expression in BPH cells*. The effect of 48 h treatment with BXL-353 (10 nM) alone or in combination with GFs (KGF and Des [1–3] IGF-I, 10 ng/ml) or T (10 nM) is reported on apoptosis (**panel A**) and Bcl-2 expression (**panel B**). Apoptotic index was obtained from *in situ* end labeling (ISEL) experiments and represents the number of stained nuclei over total cells in each of at least 5 separate fields per slide. Percentage of Bcl-2 stained cells was calculated by counting the number of immunopositive BPH cells over total cells in each of at least 5 separate fields per slide. Treatment with GFs or T significantly decreased the number of apoptotic cells (panel A), while it increased Bcl-2 positivity (panel B). Simultaneous treatment with GFs or T and BXL-353 significantly blunted the effect of both GFs and T on the number of ISEL (panel A) and Bcl-2 (panel B) positive cells. A 48 h exposure to BXL-353 induced a massive increase in apoptotic index (panel A), while Bcl-2 expression decreased (panel B). $^{*}P < 0.01$ vs. control; $^{\circ}P < 0.01$ vs. GF- or T-treated cells by one-way ANOVA and paired or unpaired Student's t tests. The data are partially derived from Crescioli *et al*., 2000; 2002; 2003.

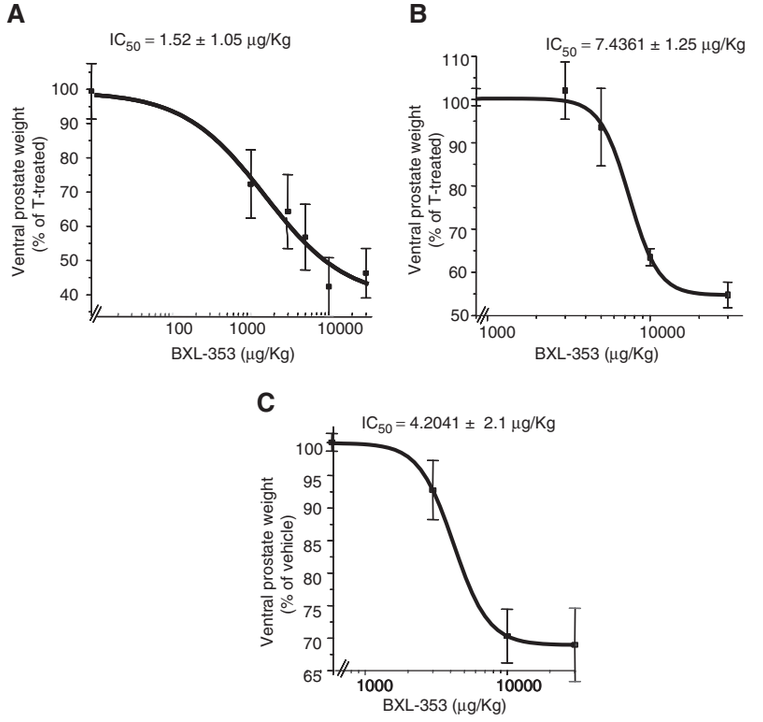

FIGURE 5 Effect of BXL-353 on rat ventral prostate weight. **Panel A**: Castrated rats were supplemented with a single injection of T enanthate (30 mg/Kg/week) and orally treated for 4 days with vehicle or increasing concentrations of BXL-353 (1–30 μg/Kg). Ventral prostate weight is expressed as % variation (mean ± SEM) of the weight of T-replaced castrated rats, in two separate experiments. ^$P < 0.05$ and *$P < 0.01$ vs T-supplemented vehicle-treated rats by one-way ANOVA and paired or unpaired Student's t tests. **Panel B**: Castrated rats were injected with T enanthate (15 mg/Kg/week) and orally treated for 5 day/week for two consecutive weeks with vehicle or increasing concentrations of BXL-353 (3–30 μg/Kg). Ventral prostate weight is expressed as % variation (mean ± SEM) of the weight of T-replaced castrated rats (n = 4). *$P < 0.01$ vs. T-supplemented vehicle-treated rats by one-way ANOVA and paired or unpaired Student's t tests. **Panel C**: Intact adult rats were orally treated for over one month (5 times/week for a total of 27 administrations) with vehicle (control) or increasing concentrations of BXL-353 (3–30 μg/Kg). Ventral prostate weight is expressed as % variation (mean ± SEM) of the weight of control rats (n = 4) (*$P < 0.01$ vs control rats). Data are derived from Crescioli *et al*., 2003.

prostate cells after sub-acute (7–14 days, castrated rats) or prolonged (1 month, intact rats) treatment with the analog. By using terminal deoxynucleotidyl transferase (TdT) mediated dUTP nick end-labeling (TUNEL), we observed the typical hallmark of nuclear fragmentation in both the epithelial and stromal cells of the BXL-353-treated prostate in all the experimental protocols studied [31]. In addition, we found that BXL-353 treatments induced a dose- and time-dependent up-regulation of clusterin gene and protein. Clusterin (CLU), or testosterone-repressed message 2 (TRPM-2), is an ubiquitous, puzzling protein expressed also in the rat prostate [73–75], which is down-regulated by androgens and up-regulated by growth arrest and cell death (see in [76]). CLU has different intracellular and extracellular functions. As an extracellular, secretory glycoprotein, it has a chaperone-like role, binding a wide range of unrelated molecules and probably clearing cellular debris. Conversely, the intracellular ~49 kDa protein, after appropriate stimuli, is transported from the cytoplasm to the nucleus, where it binds DNA helicases, as Ku70/Ku86, thereby reducing DNA repair and allowing cell death [77]. Hence, CLU is generally considered an androgen-regulated, pro-apoptotic protein. We confirmed that in the rat prostate, CLU expression was increased by castration and finasteride administration [73,75], and we found that BXL-353 induced a sustained increase in CLU gene and protein

expression [31]. Interestingly, CLU-positive cells were more apparent in the glands of BXL-353 treated rats showing the more pronounced features of involution and atrophy.

In conclusion, studies in the rat strongly support findings in human BPH cells: BXL-353, similar to finasteride, counteracts the growth promoting effect of T, by inducing growth cell arrest and apoptosis. However, BXL-353, at variance with finasteride, is not an antiandrogen. It does not bind to the AR, as demonstrated by competition studies using the synthetic androgen [^{3}H] R1881on BPH homogenates, and it does not inhibit 5α-reductase activity, as shown by the failure to interfere with DHT formation in CHO cells transfected with type 1 or type 2 5α-reductase iso-enzymes [31]. In addition, BXL-353 did not affect the gonadal or pituitary secretion of testosterone or gonadotrophin [31]. Hence it should act downstream of the AR receptor ligand interaction. The activated AR is a multiple phosphorylated protein and some of its phosphorylation sites (as Ser 650) are required for full transcriptional activity (see ref. [78]). Hence, it is possible that BXL-353 might activate the catalytic subunit of distinct families of phosphatases, therefore exerting its antiproliferative effects acting on AR-dependent signaling. Alternatively, BXL-353 may disrupt androgen-dependent GF-mediated survival pathways, thus hampering T-induced BPH cell growth.

VII. CONCLUSIONS

A large proportion of aging males develop BPH and, until recently, the only options for treatment were surgical intervention or watchful waiting. During the last 10 years, progress in medical therapy of BPH has resulted in effective treatments patterns leading to a significant improvement in the quality of life of affected patients. At present, two different classes of agents are available for BPH treatment: α-blockers and 5α-reductase inhibitors. Although sexual related side effects are more often reported with 5α-reductase inhibitors than with α-blockers [79], the reverse is true for reduction in risk of BPH-related surgeries [80]. A population-based cohort study, conducted in more than 5000 patients, receiving either α-blockers or 5α-reductase inhibitors (finasteride), showed that the incidence of BPH-related surgery was higher in α-blocker-treated patients than in 5α-reductase inhibitor-treated ones [80]. Similar results, obtained from a retrospective analysis of patients' data, demonstrated that the risk of experiencing serious complication related to BPH progression (catheterization, acute urinary retention, surgery) was significantly lower in finasteride-treated patients compared to patients using α-blockers [81]. A possible mechanism of action underlying the risk reduction of BPH-related surgery by finasteride is that 5α-reductase inhibitors, by blocking DHT formation, shrink the prostate volume, which, in turn, is shown to be itself an important risk factor for BPH progression and, consequently, BPH-related surgery [80,82]. In fact, although many variables make it difficult to predict an individual's clinical course, prostatic size is reported to be one of the most important risk factors along with age and prostatic specific antigen (PSA) value [83]. Hence, the ideal treatment for BPH should include a medication that reduces prostate volume without interfering with androgen activity. Actually, patient compliance for finasteride may be limited by consistent sexual side effects, such as decreased libido, altered sexual potency, or ejaculatory dysfunction [84], especially in men with borderline erectile function [85]. Well-tolerated calcitriol analogs, such as BXL-353, might represent such a new class of drugs, because they decrease AR-mediated prostate growth, acting downstream of the AR on the GF-mediated proliferation pathways. Based on the data reviewed here, a double-blind, placebo-controlled phase II study is currently ongoing in Italy to evaluate the effects of a nonhypercalcemic calcitriol analog in patients with BPH.

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Index

A

B

C

D

E

F

G

H

I

J

K

L

M

N

O

P

Q

R

S

T

U

V

W

X

Y

Z