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In vivo function of VDR in gene expression-VDR knock-out mice ☆

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Abstract

Vitamin D exerts many biological actions through nuclear vitamin D receptor (VDR)-mediated gene expression. The transactivation function of VDR is activated by binding 1α,25-dihydroxyvitamin D_3[1α,25$(OH)_2D_3$], an active form of vitamin D. Conversion from 25$(OH)D_3$ is finely regulated in kidney by 25$(OH)D_3$ 1α-hydroxylase[25(OH)D 1α-hydroxylase], keeping serum levels of 1α,25$(OH)_2D_3$ constant. Deficiency of vitamin D and mutations in the genes like VDR (type II genetic rickets) are known to cause rickets like lowered serum calcium, alopecia and impaired bone formation. However, the molecular basis of vitamin D–VDR system in the vitamin D action in intact animals remained to be established. In addition, the 1α-hydroxylase gene from any species had not yet been cloned, irrespective of its biological significance and putative link to the type I genetic rickets. We generated VDR-deficient mice (VDR KO mice). VDR KO mice grew up normally until weaning, but after weaning they developed abnormality like the type II rickets patients. These results demonstrated indispensability of vitamin D–VDR system in mineral and bone metabolism only in post-weaning life. Using a newly developed cloning system, we cloned the cDNA encoding a novel P450 enzyme, mouse and human 1α-hydroxylase. The study in VDR KO mice demonstrated the function of liganded VDR in the negative feed-back regulation of 1α,25$(OH)_2D_3$ production. Finally, from the analysis of type I rickets patients, we found missense genetic mutations in 1α-hydroxylase, leading to the conclusion that this gene is responsible for the type I rickets.

1. Introduction

The most biologically active form of vitamin D, 1α,25-dihydroxyvitamin D_3[1α,25$(OH)_2D_3$] has roles in a variety of biological actions such as calcium homeostasis, cell proliferation and cell differentiation to many target tissues [1,2]. Most of such biological actions of 1α,25$(OH)_2D_3$ are thought to be exerted through gene expression mediated by VDR [3]. VDR is a member of the nuclear hormone receptor superfamily and acts as a ligand-inducible transcription factor [4,5]. 1α,25$(OH)_2D_3$ is a most potent form of vitamin D and acts as a specific ligand for VDR.

1α,25$(OH)_2D_3$ is biosynthesized from cholesterol, and at the final steps two hydroxylations (hepatic 25-hydroxylation and renal 1α-hydroxylation) occur for its metabolic activation into a hormonal form [6,7]. Renal hydroxylation of 25$(OH)D_3$ is crucial for the biosynthesis, and is conducted by 25$(OH)D_3$ 1α-hydroxylase[25(OH)D 1α-hydroxylase] in the proximal tubule of the kidney [8]. It has been shown that the activity of 25$(OH)D_3$ 1α-hydroxylase is inhibited by its end product, 1α,25$(OH)_2D_3$, and activated by calciotropic peptide hormones such as calcitonin and PTH [6,7], though the molecular mechanism underlying these regulations remained unclear.

To investigate the roles of 1α,25$(OH)_2D_3$–VDR in intact animals, we generated the mice lacking VDR by

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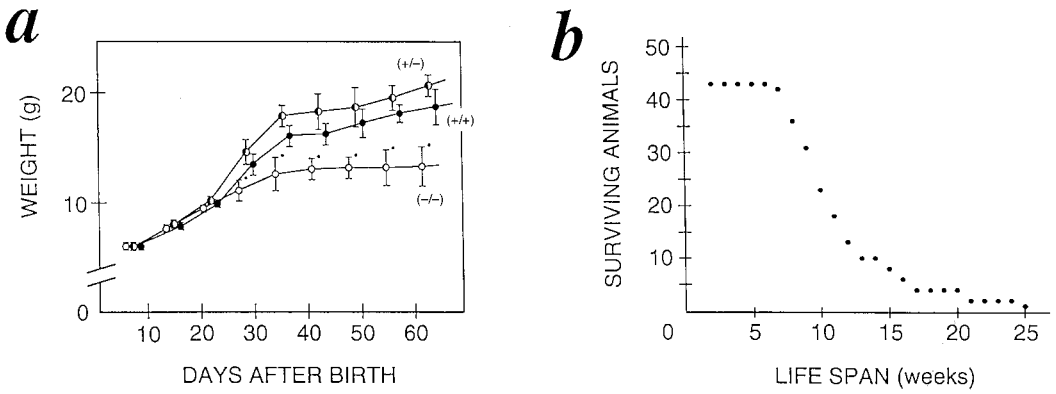

Fig. 1. Phenotypes of the $VDR^{-/-}$ mice: (a) representative growth curve of wild-type (+/+), heterozygote (+/−), and homozygote (−/−) littermates; and (b) survival rate of 43 $VDR^{-/-}$ mice.

targeted gene disruption, and analyzed the phenotype of the mice.

2. Vitamin D receptor inactivation by homologous gene targeting in mice

We generated mice deficient of VDR by gene targeting in order to investigate the function of VDR in vivo [8]. A targeting plasmid was constructed to disrupt the VDR gene by inserting a *neo*^{r} gene into the exon 2 that encodes the first Zn finger motif in the DNA binding domain essential for the biological functions of VDR. Two lines of mice heterozygous for the mutation showed no obvious defects and were interbred to generate homologous gene targeted mice ($VDR^{-/-}$).

Moreover, no bone malformation or overt phenotypic abnormalities were seen in the $VDR^{-/-}$ fetuses (data not shown), although several lines of in vitro studies have shown catabolic effects of $1\alpha,25(OH)_2D_3$ in fetal bone [9].

3. Appearance of rickets with growth retardation only after weaning in VDR KO mice

Unexpectedly, the VDR-null mutant mice did not differ from the heterozygous or wild-type littermates in growth rate (Fig. 1a) or behavior, and seemed functionally normal after birth until weaning. However, after weaning (about 3 weeks), the VDR-null mutant mice unexpectedly showed marked growth retardation, and the body weight of the null mutant mice at 10 weeks was about 50% of those of the heterozygous and wild-type mice (Fig. 1a). After weaning the VDR-null mutant mice developed rickets, and most of them died by 15 weeks due to an unknown reason (Fig. 1b). However, no overt abnormalities were found in the heterozygotes even at 6 months. By 7 weeks, all of the VDR-null mutant mice developed alopecia and poor whisker as typical features of rickets, and most of them displayed flat face with shorter nose. In the null mutant mice at 7 and 13 weeks, no apparent abnormalities were found by histological analysis in the VDR-expressing tissues other than bone and skin, including the intestine, kidney, brain, spleen. Uterine hypoplasia was found in the 10-week-old null mutant mice, though no abnormality was found in the male reproductive organs.

The observations in the VDR-null mutant mice by this study and the others [8,10] are similar to a human hereditary and recessive disease, vitamin D dependency type II [11], in which mutations in the VDR gene have been identified in several families [12], although unlike the VDR KO mice, this disease is not lethal. As these patients exhibit rickets with hypocalcemia, hypophosphatemia and elevated serum alkaline phosphatase (ALP), serum levels of these parameters were measured in the mice from 3 to 13 weeks old. During lactation (at 3 weeks), no effects of inactivation of VDR were seen in agreement with the phenotype and growth rate. However, at 4 weeks, only one week after weaning, the serum levels of calcium and phosphate were reduced with markedly elevated serum ALP activity in the VDR-null mutant mice. At this stage, no defects such as alopecia were observed. In older VDR-deficient mice, these abnormalities became more prominent. The serum level of $1\alpha,25(OH)_2D_3$ is strictly regulated to remain constant. Several enzymes, which are regulated by several factors including $1\alpha,25(OH)_2D_3$, are involved in synthesis and metabolism of $1\alpha,25(OH)_2D_3$ [13]. The activities of 25(OH)D 1α-hydroxylase and 24-hydroxylase are regulated negatively and positively, respectively, by $1\alpha,25(OH)_2D_3$. In the VDR-null mutant mice at 3 weeks, the serum levels of $1\alpha,25(OH)_2D$, 24, $25(OH)_2D$, and 25(OH)D were the same as those in the heterozygous and wild-type mice. However, a marked increase in serum $1\alpha,25(OH)_2D_3$, and a clear

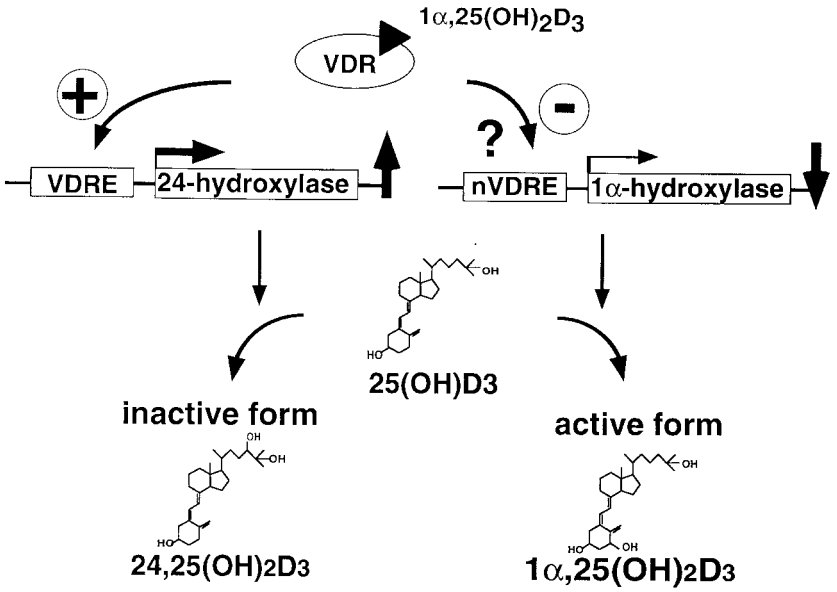

Fig. 2. A proposed molecular mechanism of regulations of $1\alpha,25(OH)_2D_3$ biosynthesis by 25-hydroxyvitamin D_3 1α-hydroxylase and $25(OH)D_3$–24-hydroxylase. The negative regulation of 1α(OH)ase gene expression by $1\alpha,25(OH)_2D_3$ did not occur in the mice lacking VDR (VDR knockout mice) [8], raises a possibility that a negative VDRE is present in the promoter of the 1α(OH)ase gene. The positive VDRE has been identified in the promoter of the $25(OH)D_3$–24-hydroxylase gene [23]. The levels of serum $1\alpha,25(OH)_2D_3$ is positively and negatively regulated through these VDREs binding liganded VDR.

reduction in serum $24,25(OH)_2D$ had developed in the VDR-null mutant mice at 4 weeks and persisted at 7 weeks, suggesting increased activity of 1α-hydroxylase and reduced activity of 24-hydroxylase.

To further test this hypothesis, the expression of the target genes for vitamin D (calbindin-D_{9K}: C9K, osteocalcin: OC, osteopontine: OPN) [14] were examined by Northern blot analysis in the mutant mice at 3 and 7 weeks. In the intestine as well as in kidney, even at 3 weeks the gene expression of C9K was clearly decreased as at 7 weeks, while no effects were observed on the intestinal gene expression of 9-*cis* retinoic acid receptor (RXRα) or cellular retinol binding protein II (CRBPII). It is generally accepted that both of OPN and OC are involved in the bone formation [15]. The inactivation of VDR caused a reduction in the gene expression of OPN, but not in OC in the mutant mice at 3 and 7 weeks. Regardless of the drastic alteration in the expression of the vitamin D target genes in the tissues, and the previous studies about the effects of $1\alpha,25(OH)_2D_3$ on immune system and response [16] no obvious difference between the null mutant and wild-type mice at 7 weeks, as well as at 3 weeks, was detected in the proportional change of immunological cell population.

4. Impaired bone formation in VDR KO mice

Severe malformation induced by the inactivation of VDR only after weaning was detected in bone. Radiographic analysis of the VDR-null mutant mice at 7 weeks revealed growth retardation with loss of bone density. In gross appearance and on X-ray analysis of tibia and fibula, typical features of advanced rickets were observed including widening of epiphyseal growth plates, thinning of the cortex, fraying, cupping and widening of the metaphysis. In addition, orderly columns of hypertrophic chondrocytes were lost and the layers of cartilage were widened with inadequate mineralization. In cancellous bone adjacent to the growth plates, marked increases in the extent and the width of osteoid seams were noted, and bone surfaces were surrounded by numerous osteoblastic cells. Interestingly, the number of osteoclasts appeared not to be reduced in the bone from the VDR-null mutant mice. These findings indicate that $1\alpha,25(OH)_2D_3$ can stimulate, but is not essential for osteoclast formation in vivo, and that the other factor(s) can induce the osteoclast formation in the absence of the $1\alpha,25(OH)_2D_3$ actions.

5. Cloning of mouse 25(OH)D 1α-hydroxylase cDNA from VDR KO mice by a newly developed cloning system, and identification as the responsible gene for the vitamin D dependent rickets type I

From the elevated levels of serum $1\alpha,25(OH)_2D$ in the VDR KO mice, it appeared that the VDR KO mice have the increased activity of 25(OH)D 1α-hydroxylase. Though the enzymatic activity of 25(OH)D 1α-hydroxylase is known over 25 years, the cDNA of

this enzyme had not yet been cloned. From the VDR KO mice, we were able to, for the first time, clone the cDNA encoding mouse 25(OH)D 1α-hydroxylase by a newly developed expression cloning method [17]. Furthermore, using this cloned mouse cDNA, we isolated the cDNA encoding human 1α-hydroxylase. Furthermore, homozygous missense mutations in this gene to lose its enzymatic activity were found in the patients suffering from vitamin D dependent rickets type I (VDDR I) [18].

6. VDR is essential for the negative regulation of the 25(OH)D 1α-hydroxylase gene by vitamin D

As $1\alpha,25(OH)_2D_3$ plays a primary role in calcium homeostasis, the renal activity is positively regulated by calcitropic hormones, responding to serum calcium levels. $1\alpha,25(OH)_2D_3$ has been well characterized as a negative regulator for the renal activity of 25(OH)D 1α-hydroxylase [19,20]. A study using VDR knock-out mice showed that one of the negative regulators, $1\alpha,25(OH)_2D_3$, acts at the transcriptional level, and this negative regulation requires the liganded-VDR [17], since no negative regulation by $1\alpha,25(OH)_2D_3$ was observed in the VDR knock-out mice. Thus, these findings indicate that the liganded VDR is essential for the negative regulation of the 25(OH)D 1α-hydroxylase gene by $1\alpha,25(OH)_2D_3$, and presence of a negative VDRE is speculated in the promoter of the 25(OH)D 1α-hydroxylase gene (Fig. 2).

7. Conclusion

7.1. Molecular basis for hereditary rickets (type I and II)

We have demonstrated here that the $1\alpha,25(OH)_2D_3$–VDR system is essential for growth, bone formation, and hair development only after weaning. The phenotype of the $VDR^{(-/-)}$ mice, but not heterozygous$^{(+/-)}$ mice, was similar to a human recessive genetic disease, vitamin D dependent rickets type II VDDR II [21]. However, in patients suffering from this disease, no null mutants for VDR have yet been reported [21] raising a possibility that the null mutation causes early lethality and/or infertility. In addition of type II hereditary rickets, the type I hereditary rickets is known as a recessive genetic disease lacking ability to convert $25(OH)D_3$ into $1\alpha,25(OH)_2D_3$ by 25(OH)D 1α-hydroxylase [22]. Using the cloned mouse 25(OH)D 1α-hydroxylase from the VDR KO mice [17], we have cloned the cDNA encoding human 25(OH)D 1α-hydroxylase, and found homozygous missense mutations in this gene in the type I rickets patients [18] (Fig. 3).

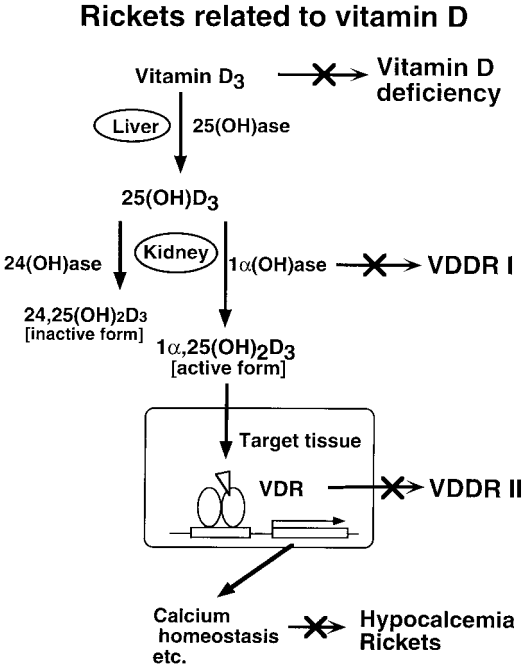

Fig. 3. Rickets related with vitamin D. The biosynthesis pathway of $1\alpha,25(OH)_2D_3$ and the mode of $1\alpha,25(OH)_2D_3$ action are illustrated. The defects in these processes cause rickets. Nutritional vitamin D deficiency, and the defect of the renal 1α(OH)ase activity by genetic mutations (VDDR I patients) result in short supply of vitamin D. The mutated VDR in the VDDR II patients is unable to respond to $1\alpha,25(OH)_2D_3$, resulting in the rickets.

7.2. Are vitamin D actions on bone direct or indirect?

Consistent with the findings in rickets patients and animals in a vitamin D deficiency state, VDR inactivations caused impaired bone formation, but unexpectedly its onset was only after weaning. Histological analysis of bone in the VDR KO mice showed that the impaired bone formation appears due to mainly an impaired mineralization, but not to reduced numbers of osteoclasts and osteoblasts. Dietary supplementation of calcium to VDR KO mice seems to significantly recover this impaired mineralization, however, it turned out that the disordered proliferation and differentiation of chondrocyte in the VDR KO mice is not significantly restored by calcium supplementation (N. Yagishita, T. Yoshizawa et al., manuscript in preparation). Taken together, we speculate that the $1\alpha,25(OH)_2D_3$–VDR system is more important for the cell proliferation and differentiation of chondrocyte, however, a further detail study is apparently required, since the other factors to control cell proliferation and differentiation of bone cells are most likely to be over- or under-produced in the VDR KO mice. A conditional VDR KO in bone, intestine and the major target tissues for vitamin D by means of the Cre-LoxP

system, which is currently under investigation in our laboratory, will clarify these problems.

References

[1] R. Bouillon, W.H. Okamura, A.W. Norman, Structure-function relationships in the vitamin D endocrine system, End. Rev. 16 (1995) 200–257.

[2] G.P. Studzinski, J.A. McLane, M.R. Uskokovic, Signaling pathways for vitamin D-induced differentiation: implications for therapy of proliferative and neoplastic diseases, Crit. Rev. Eukar. Gene Exp. 3 (1993) 279–312.

[3] H. Darwish, H.F. DeLuca, Vitamin D-regulated gene expression, Cric. Rev. Eukar. Gene Exp. 3 (1993) 89–116.

[4] D.J. Mangelsdorf, R.M. Evans, The RXR heterodimers and orphan receptors, Cell 83 (1995) 841–850.

[5] K. Ebihara, Y. Masuhiro, T. Kitamoto, M. Suzawa, Y. Uematsu, T. Yoshizawa, T. Ono, H. Harada, T. Hasegawa, S. Masushige, S. Kato, Intron retention generates α novel isoform of the murine vitamin D receptor that acts in α dominant negative way on the vitamin D signaling pathway, Mol. Cell. Biol. 16 (1996) 3393–3400.

[6] H. Reichel, H.P. Koeffler, A.W. Norman, The role of the vitamin D endocrine system in health and disease, N. Engl. J. Med. 320 (1989) 980–991.

[7] D.R. Fraser, Regulation of the metabolism of vitamin D, Physiol. Rev. 60 (1980) 551–613.

[8] T. Yoshizawa, Y. Handa, Y. Uemasu, S. Takeda, K. Sekine, Y. Yoshihara, T. Kawakami, K. Arioka, H. Sato, Y. Uchiyama, S. Masushige, A. Fukamizu, T. Matsumoto, S. Kato, Mice lacking the vitamin D receptor exhibit impaired bone formation, uterine hypoplasia and growth retardation after weaning, Nature Gen. 16 (1997) 391–396.

[9] J.B. Lian, M. Coutts, E. Canalis, Studies of hormonal regulation of osteocalcin synthesis in cultured fetal rat calvariae, J. Biol. Chem. 260 (1985) 8706–8710.

[10] Y.C. Li, A.E. Pirro, M. Amling, et al., Targeted ablation of the vitamin D receptor: an animal model of vitamin D-dependent rickets type II with alopecia, Proc. Natl. Acad. Sci. USA 94 (1997) 9831–9835.

[11] S.J. Marx, U.A. Liberman, C. Eil, et al., Hereditary resistance to 1,25-dihydroxyvitamin D, Rec. Prog. Hor. Res. 40 (1984) 589–620.

[12] M.R. Hughes, P.J. Malloy, D.G. Kieback, et al., Point mutations in the human vitamin D receptor gene associated with hypocalcemic rickets, Science 242 (1988) 1702–1705.

[13] D.R. Fraser, Regulation of the vitamin D metabolism, Physiol. Reviews 60 (1980) 556–613.

[14] M. Noda, R.L. Vogel, A.M. Craig, et al., Identification of α DNA sequence responsible for binding of the 1,25-dihydroxyvitamin D3 receptor and 1,25-dihydroxyvitamin D3 enhancement of mouse secreted phosphoprotein 1 (SPP-1 or osteopontin) gene expression, Proc. Natl. Acad. Sci. USA 87 (1990) 9995–9999.

[15] G.S. Stein, J.B. Lian, Molecular mechanisms mediating proliferation/differentiation inter-relationship during progressive development of the osteoblast phenotype, Endocr. Rev. 14 (1993) 424–442.

[16] W.F.C. Rigby, The immunobiology of vitamin D, Immunology Today 9 (1988) 54–58.

[17] K. Takeyama, S. Kitanaka, T. Sato, M. Kobori, J. Yanagisawa, S. Kato, 25-Hydroxyvitamin D3 1α-hydroxylase and vitamin D synthesis, Science 277 (1997) 1827–1830.

[18] S. Kitanaka, K. Takeyama, A. Murayama, T. Sato, K. Okumura, M. Nogami, Y. Hasegawa, H. Niimi, J. Yanagisawa, T. Tanaka, S. Kato, Inactivating mutations in the 25-hydroxyvitamin D3 1α-hydroxylase gene in patients with pseudovitamin D-deficient rickets, N. Engl. J. Med. 338 (1998) 653–661.

[19] H.L. Henry, R.J. Midgett, A.W. Norman, Studies on calciferol metabolism: Regulation of 25-hydroxyvitamin D3-1α-hydroxylase, in vivo, J. Biol. Chem. 249 (1974) 7584–7592.

[20] H.L. Henry, Regulation of the hydroxylation of 25-hydroxvitamin D3 in vivo and in primary cultures of chick kidney cells, J. Biol. Chem. 254 (1979) 2722–2729.

[21] P.J. Malloy, Z. Hochberg, D. Tiosano, J.W. Pike, M.R. Hughes, D. Feldman, The molecular basis of hereditary 1,25-dihydroxyvitamin D3 resistant rickets in seven related families, J. Clin. Invest. 86 (1990) 2071–2079.

[22] M. Labuda, K. Morgan, F.H. Glorieux, Mapping autosomal recessive vitamin D dependency type 1 to chromosomal 12q14 by linkage abalysis, Am. J. Hum. Genet. 47 (1990) 28–36.

[23] Y. Ohyama, K. Ozono, M. Uchida, T. Shinki, S. Kato, T. Suda, O. Yamamoto, M. Noshiro, Y. Kato, Identification of α vitamin D-responsive element in the 5′-flanking region of the rat 25-hydroxyvitamin D3 24-hydroxylase gene, J. Biol. Chem. 265 (1994) 10,545–10,550.