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Journal of Steroid Biochemistry & Molecular Biology 89–90 (2004) 343–345
The Journal of Steroid Biochemistry & Molecular Biology
www.elsevier.com/locate/jsbmb

Direct action of 1,25-dihydroxyvitamin D on bone: VDRKO bone shows excessive bone formation in normal mineral condition☆

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Abstract

In the present study, the direct role of Vitamin D in bone metabolism was investigated. Vitamin D has been suggested to be an important hormone for bone metabolism, but there has been little evidence that Vitamin D actively participates in this process. Here, we show the direct action of Vitamin D by transplanting the bone of the Vitamin D receptor null mutant mice (VDR−/−) to the wild-type mouse. This procedure allowed us to investigate the changes in the bone without VDR in the normal humoral environment. Unexpectedly, the volume and the density of the VDR−/− bone transplanted to the wild-type mouse were significantly increased compared with the control (wild-type bone transplanted to the wild-type mouse). We show that Vitamin D has key roles in bone metabolism negatively.

Keywords: Vitamin D; Wild-type mouse; Vitamin D receptor null mutant mouse; Transplantation

1. Introduction

Vitamin D has been suggested to be an important hormone for calcium homeostasis and bone metabolism [1,2], but there has been little evidence that Vitamin D directly regulates bone formation. The direct role of Vitamin D in bone formation is still controversial. It was reported that Vitamin D increased bone remodeling via stimulating bone cells [3,4]. In contrast, it was also reported that Vitamin D did not need bone formation or calcification [5,6].

The Vitamin D receptor null mutant mouse (VDR−/−) has provided new insights into Vitamin D metabolism and its role in vivo [7]. Calcium-supplement experiments aimed at establishing physiological direct functions of VDR in many organs including bone have been inconclusive owing to the essential roles of calcium in biological function. Although calcium supplementation showed an apparent cure of rickets [8,9], we could not exclude a compensatory mechanism such as hyperparathyroidism in this process. To evaluate the direct action of Vitamin D on the bone without an influence of calcium homeostasis, it was necessary to investigate the bone of the VDR−/− under a normal environment. To this aid, we performed bone-transplantation of the VDR−/− to the wild-type mouse.

2. Materials and methods

2.1. Animals

VDR null mutant mice were generated by gene targeting as described previously [7]: the locus targeted for the disruption of the VDR gene included exon 2, and the mutant locus contained the neomycin resistant gene. Mice were weaned at 3 weeks of age, and were then fed distilled water and a chow diet ad libitum, (MF, Oriental Yeast, Tokyo, Japan; ingredients: 11.1 mg/g calcium, 8.3 mg/g phosphorous, 1.08 IU/g Vitamin D_3). The mice were maintained under specific pathogen-free conditions with a 12 h light, 12 h dark cycle. They were bred as heterozygotes. The VDR genotypes were determined by the previously reported methods [10]. The studies were reviewed and approved by the Institutional Committee of Animal Care and Use of Okayama University Graduate School of Medicine and Dentistry.

2.2. Bone transplantation

The femur and the calvaria prepared from the 2-week-old VDR−/− males were transplanted into the back muscle of the wild-type males and VDR−/− males. The femur and calvaria from the wild-type mice were also transplanted to the wild-type mouse and VDR−/− mice. VDR−/− bone was transplanted to one side of the back and wild-type bone was transplanted to the other side of the back in the same host at the same time. The parents were the same pair both

☆ Presented at the 12th Workshop on Vitamin D (Maastricht, The Netherlands, 6–10 July 2003).

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doi:10.1016/j.jsbmb.2004.03.021

in the donor and the host and the host mice were 6 weeks old. The host mice were maintained under the regular dietary conditions, and the transplanted bones were retrieved after 2, 3 or 4 weeks.

2.3. Analysis of skeletal morphology

Bone radiographs of the transplanted femur were taken with a micro-focus X-ray apparatus (25 kV, 80 μA, 5 s, μFX-1000 digital micro-radiography system; Fuji Photo Film Co., Ltd.). The projected bone area and the density of the femur were analyzed with an imaging analyzer (BAS-2000 II, Fuji Photo Film Co., Ltd.). For Villanueva–Goldner staining, transplanted femurs were excised, fixed with 70% ethanol, embedded in methyl methacrylate, and sectioned into 6 μm slices.

2.4. Serum chemistries

Calcium levels were measured using the *o*-cresol phthalein complexion method (Wako, Osaka, Japan). Phosphorous levels were measured using the *p*-methylaminophenol method (Wako).

2.5. Statistical analysis

Values are given as the means±S.E.M. Statistical analysis was performed using unpaired Student's *t*-test and ANOVA, followed by Fisher's protected least significant difference. $P < 0.05$ was considered significant.

3. Results

3.1. Serum calcium, phosphorous, and ALP activity in the wild-type host mouse

Serum calcium, phosphorous, and ALP activity in the wild-type host mouse were measured at pre-transplantation, and at 2, 3 and 4 weeks after transplantation. These parameter levels are shown in Table 1. There were no significant differences between the transplantation periods. The serum calcium and ALP levels were maintained constant during the transplantation period. The serum phosphorous level was slightly decreased as physiological natural course.

Table 1
Serum calcium, phosphorous, and ALP activity in the wild-type host mouse

Transplanted periods	Ca	P	ALP
Pre	8.31 ± 0.26	8.18 ± 0.68	9.34 ± 0.53
2 weeks	8.38 ± 0.18	7.72 ± 0.23	9.82 ± 0.39
3 weeks	8.17 ± 0.17	7.57 ± 0.38	9.63 ± 0.38
4 weeks	8.37 ± 0.27	6.93 ± 0.35	9.10 ± 0.45

Values are given as means ± S.E.M. (Ca, mg/dl; P, mg/dl; ALP, nmol *p*-nitrophenol/30 min). $n = 4$–5. Serum calcium, phosphorous, and ALP activity in the VDR−/− were 5.36 ± 0.25 mg/dl, 5.26 ± 0.37 mg/dl, and 19.05 ± 1.48 nmol *p*-nitrophenol/30 min, respectively. During the transplanted period, serum calcium, phosphorous, and ALP activity in the wild-type host mouse was not changed.

Table 2
The density and the projected area of the transplanted femur (transplanted period: 4 weeks)

KO-WT	WT-WT	KO-KO	WT-KO	
Density (%)	148 ± 29*	100 ± 5	58 ± 2*	35 ± 2*
Area (%)	104 ± 2	100 ± 2	82 ± 2	59 ± 2*

The images of the radiographs by micro-focus X-ray of the transplanted femur were analyzed using an imaging analyzer system. Values are given as the means ± S.E.M. (the mean values of the density and area in the WT-WT bone were expressed as 100% and those of KO-WT, KO-KO, and WT-KO bones were expressed as a relative value to the WT-WT bone). KO-WT, the bone of the VDR−/− transplanted to the VDR+/+ mouse; WT-WT, the bone of the VDR+/+ transplanted to the VDR+/+ mouse; KO-KO, the bone of the VDR−/− transplanted to the VDR−/− mouse; WT-KO, the bone of the VDR+/+ transplanted to the VDR−/− mouse.
* $P < 0.01$ vs. WT-WT; $n = 4$.

3.2. Analysis of the transplanted bone

Four weeks after the transplantation, bone radiographs of the transplanted femur were taken and the density of the femur was analyzed using a micro-focus X-ray apparatus, and the results obtained are shown in Table 2. The density of the bone of the VDR−/− transplanted to the wild-type mouse (KO-WT) was significantly higher than that of the wild-type bone transplanted to the wild-type mouse (WT-WT). The density of the VDR−/− bone transplanted to the VDR−/− (KO-KO) was low. The density of the wild-type bone transplanted to the VDR−/− (KO-WT) was markedly decreased compared with the WT-WT. The bone area showed a similar trend as the bone density.

The histology by Villanueva–Goldner staining showed that mineralized bone tissue was significantly increased in the KO-WT compared with the WT-WT. On the other hand, the amount of osteoid tissue was increased in the bone of the KO-KO. The WT-KO, mainly consisted of fibrous tissue, was fragile and did not show organized bone structure.

The calvaria at 2 weeks old of the VDR−/− and the wild-type mice were transplanted to the wild-type mouse for 2 weeks. The thickness of the calvaria of KO-WT was 180.5 ± 8.0 μm. This was 2.7-fold greater compared with the WT-WT.

4. Discussion

Bone transplantation caused radical changes in the original bones. Wild-type femur transplanted to the VDR−/− mice (WT-KO) did not show organized bone structure. The density was markedly decreased to 35% of that of the controls (wild-type bone transplanted to the wild-type

mouse; WT-WT). High levels of 1,25-dihydroxy Vitamin D_3[1,25$(OH)_2D_3$] and PTH in the humoral environment of the VDR−/− caused the decreased bone mineral density in the WT-KO. The histology revealed that bone resorption was increased and the demineralized bone area was replaced by fibrous tissue in the WT-KO. The increased bone resorption in the WT-KO suggested that the transplantation procedure did not destroy the cells related to bone resorption such as osteoclasts, short live cells and its precursor.

The density of the KO-WT was markedly increased (1.48-fold greater than the control). Mineralized bone tissue was also significantly increased in the bone compared with the controls. The histology showed that increased bone mass was normal bone tissue not abnormal pathological mineralization. The VDR−/− and wild-type bones were transplanted to the same wild-type mouse at the same time, therefore, both bones were under the same humoral condition. No abnormal changes were observed in the serum levels of the systemic humoral environment by the transplantation procedure. In the normal humoral environment, the VDR−/− bone increased in density, in mineralized bone area and in size. There was one study that reported demineralized rachitic bone implanted into normal host rats resulted in bone formation similar to that seen for normal bone implants [11]. The present results suggested that there was no difference between the bone matrix of the VDR−/− and the wild-type and the increasing of bone formation in the KO-WT was not caused by the original difference in the bone matrix.

The thickness of the calvaria of KO-WT was 2.7-fold greater compared with the WT-WT. This result suggested that the lack of Vitamin D function in the bone caused increased membranous ossification in the normal environment.

In this study, a simple and primitive procedure, transplantation of the VDR−/− bone to a wild-type mouse, allowed us to investigate the VDR-dependent action of Vitamin D to whole bone. In the present findings, the lack of VDR caused increased bone formation. These findings provide the first direct evidence that Vitamin D is essentially a negative regulating factor in bone formation.

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