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Review

Vitamin D metabolites in captivity? Should we measure free or total 25(OH)D to assess vitamin D status?

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ABSTRACT

There is general consensus that serum 25(OH)D is the best biochemical marker for nutritional vitamin D status. Whether free 25(OH)D would be a better marker than total 25(OH)D is so far unclear. Free 25(OH)D can either be calculated based on the measurement of the serum concentrations of total 25(OH)D, vitamin D-binding protein (DBP), albumin, and the affinity between 25(OH)D and its binding proteins in physiological situations. Free 25(OH)D can also be measured directly by equilibrium dialysis, ultrafiltration or immunoassays. During the vitamin D workshop held in Boston in March 2016, a debate was organized about the measurements and clinical value of free 25(OH)D, and this debate is summarized in the present manuscript. Overall there is consensus that most cells apart from the renal tubular cells are exposed to free rather than to total 25(OH)D. Therefore free 25(OH)D may be highly relevant for the local production and action of $1,25(OH)_2D$. During the debate it became clear that there is a need for standardization of measurements of serum DBP and of direct measurements of free 25(OH)D. There seems to be very limited genetic or racial differences in DBP concentrations or (probably) in the affinity of DBP for its major ligands. Therefore, free 25(OH)D is strongly correlated to total 25(OH)D in most normal populations. Appropriate studies are needed to define the clinical implications of free rather than total 25(OH)D in normal subjects and in disease states. Special attention is needed for such studies in cases of abnormal DBP concentrations or when one could expect changes in its affinity for its ligands.

Contents

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1. Introduction

The free hormone hypothesis claims that only hormones which are not bound to high-affinity carrier proteins are free to enter cells and exert biological activity [1]. This fundamental concept has been well established for other hormones, such as sex steroid and thyroid hormones [1–3]. Similar to steroid and thyroid hormones, vitamin D is highly lipophilic and has protein carriers that help maintain circulating serum stores. The majority of circulating vitamin D (25(OH)D) is tightly bound to DBP (85–90%), an abundant circulating α-globulin produced by the liver. Approximately 10–15% of serum 25(OH)D is bound to albumin. The binding constants of DBP and albumin are ∼1000-fold different, with albumin being the weaker carrier [4]. Less than 0.1% of vitamin D circulates freely [4,5] and measurement of free 25(OH)D and 1,25 $(OH)_2D$ is technically difficult due to their low concentrations and physico-chemical behavior.

DBP, added to cultures of keratinocytes, monocytes or osteoblasts, or to kidney homogenates or bone tissue cultures substantially inhibits cellular uptake and actions of vitamin D metabolites [6–9]. However, DBP-null mice maintain normal calcium homeostasis when fed a vitamin D-replete diet and have no evidence of bone disease despite having extraordinarily low levels of total circulating 25(OH)D and 1,25$(OH)_2D$, approximately 1% of wild type controls [8,10]. In addition, in DBP-null mice, vitamin D is more rapidly taken up by the liver, and 1,25$(OH)_2D$ had a more rapid effect on calbindin induction in the intestines. However, the half life of these metabolites was much shorter in the DBP null mice, urinary losses of 25(OH)D were greater, and when placed on a vitamin D deficient diet the mice developed evidence of vitamin D deficiency more rapidly (secondary hyperparathyroidism and characteristic bone changes) [10]. These in vivo observations in DBP null mice largely supported the idea that free 25(OH)D and 1,25$(OH)_2D$ concentrations are biologically more important than their total concentrations, with DBP providing a circulating storage function for these metabolites.

In some types of cells it has been shown that specific serum protein carriers for these hormones may bind to and be transported across the cell membrane, thereby potentially enabling uptake of their hormone cargo [11,12]. This allows such cells to have access to the total hormone (bound and free) concentration. The best example is the renal tubular cell which expresses megalin and cubilin, together creating a cell surface receptor complex which acts to internalize DBP and its bound ligands. This megalin-cubilin protein complex functions as a cargo receptor and transport system for a large number of proteins, including but not limited to DBP and the bound vitamin D metabolites. This mechanism allows the kidney to recover essential proteins and their ligands and is also crucial for the reabsorption of DBP and its bound vitamin D metabolites from the glomerular filtrate [13]. This explains the greater urinary losses of the vitamin D metabolites in DBP null mice [10] or in many cases of nephrotic syndrome [14]. Some other tissues (including the parathyroid gland) express megalin, although its expression is far from universal and is usually low outside the kidney [15,16]. Whether or not the expression of megalin/cubilin is expected to distinguish which cells will be responsive to DBP-bound vitamin D metabolites versus those which are only capable of responding to the free metabolite remains for future investigation.

The *GC* gene encoding DBP has several genetic polymorphisms that may alter its vitamin D binding affinity and carrying capacity. The three most common variants of DBP (also known as GC globulin) are GC1F, GC1S, and GC2. Each variant is characterized by a different combination of two single nucleotide polymorphisms (rs4588 and rs7041) resulting in two amino acid substitutions and differing glycosylation patterns [17] (Table 1, Fig. 1).

There is a general belief that serum (total) 25(OH)D is the best marker of the vitamin D status. A large number of people around the world have low serum 25(OH)D concentrations which may increase their risk for skeletal and possibly also for extra-skeletal diseases and risks [18,19]. More recently there have been several studies suggesting that unbound (free or bioavailable[1]) 25(OH)D concentrations may be a better marker for several outcomes (bone, PTH or other extra-skeletal end points) than total 25(OH)D. This question attracted much attention when Powe et al. [20] reported that African Americans had much lower DBP concentrations (more than 50% lower than US Caucasians). As a result, despite their much lower total 25(OH)D concentration, their calculated free or bioavailable 25(OH)D was equal or even slightly higher than in Caucasians. This raised the question whether vitamin D supplementation of African Americans (as recommended by IOM 2010 and other guidelines) was required. More importantly, these observations questioned whether total 25(OH)D levels were the best marker of vitamin D status across different races or ethnic groups. Although several groups confirmed low DBP levels in African Americans [21–24] using the monoclonal R&D assay, questions were rapidly raised as to whether the monoclonal R&D assay used in these studies could be relied upon to measure polymorphic DBP as present in the serum of subjects of different races or DBP/GC genotypes [25]. The organizers of the vitamin D workshop 2016 (Boston, March 2016) invited Roger Bouillon to chair a debate with 3 experts discussing the question of how to reliably measure free 25(OH)D and DBP, summarize the existing literature with regard to 25(OH)D and racial/genetic differences and whether or not free 25(OH)D estimations are better measurements of vitamin D status than total 25(OH)D.The key questions to be answered by the group are presented in Table 2. Moreover the group was asked to identify key questions to be addressed in future studies. The present manuscript summarizes this debate and its major conclusions.

2. Vitamin D status in captivity: to free or not to free? (R. Thadhani)

Although DBP serves as an excellent high affinity reservoir for serum vitamin D metabolites, the tight affinity also implies that bound vitamin is not available for diffusion into target tissues

[1] Free 25(OH)D = unbound to whatever serum protein. Bioavailable 25(OH)D: not bound to high affinity binding protein (=free 25(OH)D + albumin bound 25(OH)D).