Clinica Chimica Acta 484 (2018) 7–13

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/cca

A monoclonal antibody sandwich ELISA for vitamin D-binding protein (VDBP) is unaffected by Gc-globulin phenotype peptides and actin and demonstrates reduced levels in sepsis and non-sepsis intensive care patients

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ARTICLE INFO

Keywords:
Vitamin D-binding protein
Gc-globulin
ELISA
Monoclonal antibodies
Vitamin D3

ABSTRACT

The measurement of vitamin D-binding protein (VDBP) by immunoassay has been confounded by variable antibody recognition of the Gc1s, Gc1F and Gc2 phenotypes. This has led to spurious conclusions regarding vitamin D status in different ethnic groups. In order to overcome these problems there is a requirement for VDBP antibodies that are unaffected by phenotype status. Here we report the generation and testing of three monoclonal antibodies to VDBP which recognise linear epitopes and are unaffected by vast molar excesses of synthetic peptides spanning these phenotypic domains. These IgG1 kappa antibodies were purified and biotinylated to allow suitable pairings to develop a sandwich ELISA for circulating VDBP. The VDBP ELISA is unaffected by actin and confirms that VDBP levels are significantly reduced in sepsis patients and non-sepsis intensive care patients compared to normal healthy subjects. Levels of VDBP along with total 25OH vitamin D3 can be used to calculate free 25OH vitamin D3 levels and these compare well with consensus values determined independently. The VDBP ELISA meets acceptable performance criteria and as such can be used in conjunction with total 25OH vitamin D3 to determine the free 25OH vitamin D3 status in various cohorts.

1. Introduction

It is becoming accepted that vitamin D status cannot be accurately assessed by only determining the total circulating level of 25OH vitamin D3 and, like other hormones, levels of their binding proteins in plasma are also relevant. The vast majority of 25OH vitamin D3 is bound to VDBP, or Gc-globulin, with a lesser amount bound to albumin resulting in < 0.1% free [1] and various algorithms can be used to estimate the free hormone [2]. This is illustrated by studies examining the vitamin D status of black Americans compared to white Americans. African Americans generally have superior bone health compared to their white counterparts [3] despite having lower circulating levels of total 25OH vitamin D3 [4]. Powe et al. [5] attempted to unravel this paradox and demonstrated that levels of VDBP were lower in African Americans thereby leading to equivalent levels of calculated free 25OH vitamin D3 in the two groups. However their study was criticized on the basis that the monoclonal antibodies used to measure VDBP differentially recognised Gc-globulin phenotypes with poorer recognition of the Gc1f phenotype which is more common in African Americans [6]. For this reason it has been established that one monoclonal antibody based VDBP immunoassay shows lower levels of VDBP in black participants compared to whites whereas some polyclonal antibody VDBP immunoassays show similar levels between the two groups [7]. More recently this has been confirmed using LC tandem MS with peptides designed to identify the three common phenotypes of VDBP [8]. These phenotypes are designated Gc1f, Gc1s and Gc2 and, apart from variable antibody recognition may associate with disease susceptibility which may reflect its diverse biological roles [9,10]. These common phenotypes have been reported to have different binding affinities to 25OH vitamin D3 [11]. However it appears that reported binding affinities for 25OH vitamin D3 are relatively minor between these phenotypes and many investigators correct neither for VDBP genotype nor binding affinity differences when calculating free 25OH vitamin D3 levels as some studies suggest it makes little difference [7,12]. On balance it would appear that an important aspect of VDBP determination would be the use of antibodies which do not bind epitope sequences within the Gc1f, Gc1s or Gc2 domains and thereby do not contribute to possible variable antibody recognition of Gc-globulin phenotypes.

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https://doi.org/10.1016/j.cca.2018.05.034
Received 4 April 2018; Received in revised form 14 May 2018; Accepted 15 May 2018

Apart from binding vitamin D, VDBP has other diverse biological functions [13] and is known to sequester actin monomers following tissue injury and this is associated with a conformational change [14] and reported to inhibit the binding of some monoclonal antibodies [15]. Hence another important aspect of VDBP determination by immunoassay is the use of VDBP antibodies without this property.

Here we report the generation of three monoclonal antibodies against human VDBP whose linear epitopes are unaffected by the presence of excess actin. Immobilised peptides spanning the Gc1f, Gc1s and Gc2 domains do not bind the monoclonal antibodies and the addition of vast molar excesses of these soluble peptides over authentic Gc-globulin do not affect antibody binding. Two of these antibodies have been paired to provide a 2-site sandwich ELISA for Gc-globulin and show reduced levels in sepsis and intensive care patients. Furthermore calculated free 25OH vitamin D3 levels show good agreement with directly measured free 25OH vitamin D3 independently determined using the DIAsource kit. We conclude that this ELISA can used to determine Gc-globulin levels in various patient cohorts and along with total 25OH vitamin D3 can be used to calculate free 25OH vitamin D3 levels.

2. Materials and methods

2.1. Monoclonal antibodies

Three female BALB/c mice were immunized intraperitoneally with 10 μg of purified human Gc-globulin (Fitzgerald Industries, Acton, MA, USA) in complete Freunds adjuvant at 4-week intervals. One week after the 4th injection, spleens were excised and fused with FOX-NY myeloma cells at a ratio of 5:1 as described previously and plated out in 96 well culture plates [16]. Supernatants were screened, after 12 days culture, using Gc-globulin coated microtitre plates (0.05 μg/well in phosphate-buffered saline, PBS). They were coated a few days in advance and, following coating, plates were washed four times with PBS containing 0.1% Tween-20 (v/v) and "blocked" with assay buffer (PBS containing 0.1% Tween-20 (v/v) and 0.1% gelatine (w/v)), 150 μL/well for 5–10 min at 20 °C. Blocked plates were then emptied by inversion and 50 μL/well of assay buffer was added. Supernatants (50 μL) from the 96 well hybridoma culture plates were then added and incubated for 60 min at 20 °C. The plates were then washed four times and sheep antimouse Ig-peroxidase added (100 μL/well at 1:1000 in assay buffer) for 30 min at 20 °C after which the plates were washed and substrate added. Substrate was prepared by the addition of 600 mL aqueous solution containing 8.2 g anhydrous sodium acetate and 3.6 g citric acid to 400 mL of methanol containing 270 mg of tetramethylbenzidine (TMB). Five hundred microliters of 30% H_2O_2 was finally added and the substrate stored in a dark bottle at room temperature. Colour development was terminated by the addition of 100 μL of 1.0 M HCL/well and the absorbance was read at 450 nm on a BMG Fluostar Galaxy (BMG Technologies, Germany). To avoid evaporation losses, plates were covered for all steps preceding the addition of substrate. Positive hybridomas were cloned twice by limiting dilution and then grown in 200 mL culture flasks. Immunoglobulin class and subclass were determined using an isotyping kit (Bio-Rad Laboratories, Hercules, CA, USA).

2.2. Purification and biotinylation of antibodies

Three Gc-globulin monoclonal antibodies were cloned and all were isotyped as IgG1 kappa. They were therefore purified from culture supernatants using Affi-Gel Protein A gel (Bio-Rad Laboratories) and used to either coat ELISA plates or were biotinylated. For biotinylation the purified antibodies were dialysed against PBS and biotinylated using EZ-Link Sulpho-NHS-biotin (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instructions.

2.3. Antibody pairing experiments

Serial dilutions of purified non-biotinylated Gc-globulin monoclonal antibodies in PBS from each of the 3 clones were coated overnight to the wells of microtitre plates (0.4–0.0125 μg/well). The wells were then "blocked" with assay buffer and emptied by inversion followed by the addition of 100 μL/well of human serum diluted 1:5000 in assay buffer for 30 min at 20 °C. The plates were then washed and dilutions (1:500–1:4000) of biotinylated Gc-globulin monoclonal antibodies in assay buffer added for a further 30 min at 20 °C. This incubation comprised a series of cross-over experiments which covered all combinations of immobilised and biotinylated Gc-globulin antibodies. The plates were again washed and streptavidin-HRP (1:1000 in assay buffer) added for a 30 min at 20 °C after which the plates were washed and TMB substrate added with further processing as described above. This strategy allowed the optimal pairing of antibodies for the development of a 2-site ELISA for Gc-globulin.

2.4. Gc-globulin sandwich ELISA

The optimal 2 site ELISA comprised coating the microtitre plate with 0.3 μg of antibody 3D2/well in PBS overnight followed by "blocking" in assay buffer. The assay buffer was emptied by inversion and then either authentic Gc-globulin standard (Fitzgerald) added in increasing doses from 0 to 8.62 nmol/L or plasma samples added (1:5000 in assay buffer), all in duplicate, 100 μL/well for 30 min at 20 °C. Following washing the plates were incubated with 0.05 μg of biotinylated Gc-globulin monoclonal antibody C8 in assay buffer/well for a further 30 min at 20 °C after which the plate were washed and streptavidin-HRP added for the final incubation with further processing as described above.

2.5. SDS-PAGE western blotting

Vertical SDS-PAGE of purified Gc-globulin (0.1 μg/lane) was carried out in 10% polyacrylamide gels in the presence of mercaptoethanol. Following transfer to nitrocellulose the membrane was "blocked" using 5% skim milk powder (overnight) and further blocked in Tris-buffered saline (TBS); (0.015 M Tris, 0.15 M NaCl, pH 7.4) containing 0.01% Tween 20 and 1% BSA. Following washing in TBS containing Tween 20 membranes were incubated with Gc-globulin monoclonal antibody supernatants from clones 3D2, 4F10 and D8 (1:100 in TBS blocking buffer) for 1 h at 20 °C and after washing with antimouse IgG-peroxidase (1:5000 in TBS blocking buffer). The membranes were finally washed and the immunoconjugates visualised using TMB precipitating substrate.

2.6. Synthetic peptides

21mer synthetic peptides corresponding to the Gc1f, Gc1s and Gc2 phenotypes and amino acids 409–428 each with a C-terminal cysteine were synthesised by Mimotopes Pty Ltd., Vic, Australia. The sequences are shown in Fig. 1 with the variant amino acids in bold and underlined. The synthesised sequences spanned the variant amino acids by at least 7 amino acids in both the N-terminal and C-terminal directions to ensure

Gc1f RLKAKLPDATPTELAKLVNK-C

Gc1s RLKAKLP**E**ATPTELAKLVNK-C

Gc2 RLKAKLPDATP**K**ELAKLVNK-C

Fig. 1. Amino acid sequences of the predominant Gc-globulin phenotypes. The 21mer synthesised peptides span amino acids 409–428 of Gc-globulin each with a C-terminal cysteine. Variant amino acids are bold and underlined.