Studies on Vitamin D Binding Protein in the Nephrotic Syndrome

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We studied the properties of vitamin D binding protein in plasma and urine from nine patients with nephrotic syndrome. Samples were incubated with 25-[3H]hydroxyvitamin D₃, after which we determined binding capacity and apparent dissociation constants. Binding capacity was markedly less in plasma from patients with nephrotic syndrome than that from normal subjects, but binding affinity was unchanged. Specific binding of 25-hydroxy[3H]vitamin D₃ could be demonstrated in urine from all the nephrotic patients, and sucrose density-gradient analysis of these urines revealed a single binding peak with sedimentation characteristics similar to those of vitamin D binding protein in plasma.

Additional Keyphrases: 25-hydroxyvitamin D₃ \textbf{\textbullet} renal disorders \textbullet} transport proteins \textbullet} urine

Vitamin D and its metabolites circulate in human plasma bound to a transport protein, vitamin D binding protein (DBP). Patients with nephrotic syndrome commonly have hypocalcemia, which is generally attributed to hypoalbuminemia. Such patients reportedly have low concentrations of 25-hydroxyvitamin D (250HD), the major circulating form of vitamin D (1). This observed decrease may be due to the loss of protein-bound vitamin D metabolites in urine (2), which may contribute to the development of hypocalcemia. Although previous workers have demonstrated the urinary excretion of DBP in nephrotic syndrome and have quantified this loss by the technique of radial immunodiffusion (2, 3), no studies have been reported on the binding properties of DBP in plasma and urine from nephrotic patients. We have, therefore, compared the 250HD₃ binding properties of plasma and urine from such patients with those of normal subjects and of patients with vitamin D deficiency.

Materials and Methods

Subjects

We studied nine patients with nephrotic syndrome (five women and four men, ages 21 to 75). All had marked proteinuria (3.5–34 g/24 h) and none was receiving steroids or vitamin supplements at the time of the study. Plasma and 24-h urine specimens were stored at –20 °C until analysis. Plasma specimens were also obtained from 12 normal control subjects (ages 19 to 49 years) and from nine patients with vitamin D deficiency.

Materials

250HD₃ was a gift from Dr. M. Uskokovic, Hoffmann-La Roche, Nutley, NJ. 25-Hydroxy(23,24(n)-)[3H]cholecalciferol, specific activity 85 Ci/mmole, was obtained from Amershams International, Amersham, Bucks, U.K. Before use, we decreased the specific activity by 25% by adding radio-inert 250HD₃.

Procedures

Binding assay. We diluted plasma samples 1000- to 2000-fold with phosphate buffer (50 mmol/L, pH 7.5); urines were diluted 100- to 300-fold. [3H]250HD₃ (0.625 to 10 nmol/L final concentration) in 10 μL of ethanol was added to 10 × 63 mm glass test tubes. Samples for estimating nonspecific binding received a 200-fold molar excess of nonlabeled 250HD₃ that had been evaporated under nitrogen before the radioactive 250HD₃ was added. The diluted plasma or urine was added to give a final volume of 200 μL, then incubated at 4 °C. After 2 h we added 25 μL of dextran-coated charcoal suspension: 10 g of charcoal (Norit GS X) and 1 g of dextran (Koch Light, M, 60 000–90 000) in 200 mL of the phosphate buffer. After a further 15-min incubation at 4 °C, with occasional stirring on a vortex-type mixer, the tubes were centrifuged at 3000 rpm (4 °C) and 100 μL of the supernatant was removed for measurement of radioactivity in 3 mL of aqueous counting scintillant (Aqua Luma: Limac BV, The Netherlands) in a scintillation counter (LKB Minibeta). By preparing Scatchard plots from the results (4), we calculated the apparent equilibrium dissociation constant (Kₐ) and maximal binding capacity (Nₐmax).

Sedimentation analysis. We incubated 200 μL of diluted urine or plasma for 2 h at 4 °C with 10 nmol of 25[3H]250HD₃ per liter, with and without radio-inert 250HD₃. After charcoal treatment to remove free steroid, aliquots were layered onto linear gradients of 50 to 200 g/L sucrose in phosphate buffer (4.2 mL total volume). We then ultracentrifuged the samples (147 000 × g maximum) for 22 h at 4 °C in a SW 50.1 rotor (Beckman) and collected fractions. The bovine serum albumin (4.4S) and human gamma globulin (6.8S), assayed with Coomassie Brilliant Blue protein reagent (5), were used as markers.

Statistics. The significance of differences between means was determined by using Student’s t-test.

Other procedures. We measured 250HD in plasma by a modification of the competitive protein binding assay of Haddad and Chyu (6), having first extracted the lipids from plasma and then purified them chromatographically. The between-assay coefficient of variation for a sample containing 40 nmol of 250HD₃ per liter was 15%. We determined calcium, creatinine, and total alkaline phosphatase activity with an AutoAnalyzer (Technicon) and albumin by a bromcresol purple dye-binding method (7). To measure the total protein of 24-h urines, we used the method of Pese and Strande (8). Plasma calcium concentration was adjusted for albumin measured by bromcresol purple method² as described by J. Nisbet and D. Oleesky (personal communication).

Results

Subjects. All patients with nephrotic syndrome were hypalbuminemic (mean 17 g/L), hypocalcemic (mean 1.86 mmol/L), and had heavy proteinuria (3.5 to 34 g/24 h). The concentration of creatinine in plasma was within the normal reference limits for all except patients 7 and 9. The

*Adjusted calcium (mmol/L) = total calcium (mmol/L) + 0.016 × (40 – albumin (g/L)).
concentration of 25OHD in plasma ranged from 3 to 45 nmol/L (mean 16 nmol/L). Our findings are summarized in Table 1.

**Binding of 25OHD₃.** To determine the 25OHD₃ binding properties of plasma from 12 normal individuals, we incubated diluted samples with increasing concentrations of 25(³H)OHD₃. The binding sites approached saturation at 8 nmol of 25(³H)OHD₃ per liter, whereas nonspecific binding increased linearly with increasing ligand concentration (Figure 1A).

Scatchard analysis of specific binding data yielded a linear plot (Figure 1B). When plasma from 12 normal subjects was examined, the apparent equilibrium dissociation constant (K_d) was calculated to be (1.18 ±0.11) × 10⁻⁹ mol/L (mean ± SEM) and the maximal binding capacity (N_max) 3.4 ±0.28 μmol/L.

We also studied the 25OHD₃ binding properties of plasma from nine vitamin D-deficient subjects. In this group, the mean (±SEM) 25OHD₃ concentration in plasma was 4.0 ±0.3 nmol/L, as compared with 35 ±5 nmol/L in the control group. The mean plasma calcium in the vitamin D-deficient group was 2.08 ±0.07 mmol/L.

Scatchard analysis of 25OHD₃ binding in plasma from vitamin D-deficient patients revealed a number of binding sites comparable with that in normal plasma: mean (±SEM) N_max in this group was 3.1 ±0.32 μmol/L with no change in affinity [K_d = (1.04 ±0.1) × 10⁻⁹ mol/L] (Figure 2). In contrast, 25OHD₃ binding capacity was significantly decreased in plasma from nine nephrotic patients as compared with the control group (p <0.001). Mean (±SEM) 25OHD₃ binding capacity in plasma from the nephrotic patients was 1.93 ±0.11 μmol/L (Figure 2), with no change in affinity [K_d = (1.0 ±0.08) × 10⁻⁹ mol/L].

**Urinary excretion of DBP in nephrotic syndrome.** Specific binding of 25(³H)OHD₃ was detected in urine from each of nine patients with nephrotic syndrome. Scatchard analysis of specific binding data indicated a range of binding capacities from 0.003 to 1.18 μmol/L (0.01 to 3.1 μmol/24 h, Table 1). The mean (±SEM) K_d was (1.06 ±0.08) × 10⁻⁹ mol/L.

No specific binding of 25(³H)OHD₃ could be detected in protein-free urines or in urine with protein content attributable to the presence of Bence Jones protein (data not shown).

**Sedimentation analysis.** We used the technique of sucrose density-gradient analysis to examine the binding moieties for 25OHD₃ in urine from patients with nephrotic syndrome. Normal plasma and urine from nephrotic patients were incubated with 10 nmol/L 25(³H)OHD₃ at 4 °C for 2 h.

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**Figure 1.** 25(³H)OHD₃ binding in normal human plasma diluted 2000-fold in phosphate buffer and incubated with 25(³H)OHD₃ (0.8–12.25 nmol/L). A, saturation plot; O, total binding; A, nonspecific binding; @ specific binding. B, Scatchard analysis of data for specific binding: K_d = 1.1 × 10⁻⁹ mol/L, N_max = 3.0 μmol/L. The regression line was calculated by least squares analysis (r = 0.98).

**Figure 2.** Vitamin D binding protein in plasma from normal, vitamin D deficient, and nephrotic subjects.

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**Table 1. Data for Nine Patients with Nephrotic Syndrome**

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In normal plasma, 25\(^{3}H\)OHD\(_{3}\) bound to a single peak, which sedimented at 4.1S (Figure 3). Binding of 25\(^{3}H\)OHD\(_{3}\) in urine from nephrotic patients also demonstrated a single peak of binding, sedimenting at 4.1S.

**Discussion**

Vitamin D binding protein is the specific transport protein for vitamin D and its metabolites in plasma. Competitive binding studies have shown that 25OHD\(_{3}\) has a higher affinity for DBP than does 1,25-dihydroxyvitamin D\(_{3}\), the physiologically active metabolite (9). In plasma, DBP is present predominantly as the apoprotein. The binding capacity in normal human serum reportedly is about 3 \(\mu\)mol/L (10); our findings for normal and vitamin D-deficient subjects are in agreement with this report. The concentration of circulating 25OHD in healthy controls is 20 to 100 nmol/L, and it has been calculated that in normal subjects only 2 to 3% of DBP binding sites are occupied.

In our studies the apparent dissociation constant for 25OHD\(_{3}\) was determined to be \(1 \times 10^{-9}\) mol/L, which is in good agreement with the findings of Haddad et al. (11), who reported a \(K_d\) of \(8 \times 10^{-10}\) mol/L. We also confirm that the DBP concentration in plasma is unaltered in vitamin D deficiency (12). There was no significant difference between DBP concentrations in plasma of normal controls and in the group with vitamin D deficiency. Thus DBP concentration is not dependent on vitamin D status and hence differs from other carrier proteins in serum such as retinol binding protein (13) and transcortin (14).

DBP is not present in the urine of normal subjects. However, in conditions involving increased glomerular permeability such as the nephrotic syndrome, DBP would be expected to appear in urine. The presence of immunoreactive DBP in urine from nephrotics has been reported (2, 3), and our studies extend these observations to demonstrate that the binding and sedimentation characteristics are very similar to DBP in plasma. The apparent dissociation for 25OHD\(_{3}\) in urine was determined to be \(1.06 \times 10^{-9}\) mol/L, a value very similar to that for plasma.

Sedimentation properties of urinary and plasma DBP were also comparable, both species sedimenting at ~4.1S. DBP has been shown to be present in cytosol preparations from virtually all tissues examined (15), but the sedimentation properties of DBP from these sources differ from those of plasma DBP. In cytosol preparations DBP forms a complex with a heat-labile 4S cytosolic component to produce a species sedimenting at 6.3S (16). In the present study, sucrose density-gradient analysis demonstrated that DBP in urine is likely to be the same molecular form as that in plasma. Urine from nephrotic subjects with bound 25\(^{3}H\)OHD\(_{3}\) showed a single peak of binding at 4.1S, identical with that in normal plasma. Thus DBP appears in urine as the 4.1S moiety and not as the "tissue" form, which sediments at 6.3S.

DBP was detectable in urine from all the nephrotic patients studied. Although only a minor fraction of the binding sites of DBP in plasma is occupied by vitamin D sterols, the continued loss of DBP in the urine might result in depletion of circulating 25OHD, leading to low concentrations of this metabolite in plasma. Indeed, two nephrotic patients had undetectable concentrations of 25OHD in their plasma and one (case 8) had a concentration in the mid-normal range. Although in this small study we saw no significant correlation between concentrations of 25OHD in plasma and urinary DBP, it is likely that concentrations of the metabolite in plasma will depend not only on the extent to which the metabolite is lost in the urine but also on the size of body stores of vitamin D and the duration of the disease. In our group of patients the duration of the disease ranged from one month to nine years, but we saw no direct correlation between this and 25OHD concentrations in plasma. Changes in liver hydroxylation of vitamin D to 25OHD cannot be excluded but seem unlikely.

Despite the low concentrations of 25OHD in their serum, patients with the nephrotic syndrome seldom show clinical signs of osteomalacia, and Korkor et al. (16) reported that 1,25(OH\(_{2}\))D\(_{3}\) values for the serum of their nephrotic patients were not significantly different from those for normal individuals.

We did not measure serum 1,25(OH\(_{2}\))D\(_{3}\) concentrations in the patients in our study, but this estimation would be of interest. Normal concentrations of (total) 1,25(OH\(_{2}\))D\(_{3}\) together with a subnormal concentration of DBP in plasma would imply an increase in "free" 1,25(OH\(_{2}\))D\(_{3}\). Bouillon et al. (17) have suggested that the free 1,25(OH\(_{2}\))D\(_{3}\) index remains normal in women who are taking oral contraceptives and during pregnancy, although both DBP and total 1,25(OH\(_{2}\))D\(_{3}\) concentrations in plasma are increased in these situations.

In summary, the present study extends previously reported observations that vitamin D binding protein is excreted in the urine of nephrotic patients and that urinary loss of this protein may contribute to the low concentrations of 25OHD in blood that are seen in this condition.

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**References**

1. Goldstein DA, Oda Y, Kuokka K, Masary SG. Blood levels of


