Possible involvement of vitamin D receptor gene polymorphism in male patients with ossification of spinal ligaments

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Abstract Ossification of spinal ligaments (OSL) is a common form of myelopathy characterized by heterotopic bone formation in the spinal ligaments, predominantly in men. Although the etiology of OSL is not fully understood, previous studies have strongly suggested the involvement of genetic factors in this disease. To investigate the possible involvement of vitamin D receptor (VDR) gene polymorphism in Japanese male patients with OSL, we analyzed: (a) the VDR genotype defined by BsmI polymorphism in patients with obvious OSL and controls; and (b) the effect of 1,25-dihydroxyvitamin D3 on alkaline phosphatase (ALP) activity of spinal ligament cells derived from patients without OSL. With regard to the VDR genotype, of the patients with OSL (n = 27), none had the BB genotype (0%), one had the Bb genotype (4%), and 26 had the bb genotype (96%). In the control group (n = 97) three had the BB genotype (3%), 18 had the Bb genotype (19%), and 76 had the bb genotype (78%). As a result, the B allele frequency in patients with OSL (2%) was significantly lower than in controls (12%). 1,25-Dihydroxyvitamin D3, at concentrations of 10^-9 and 10^-8M, significantly increased ALP activity of the ligament cells (n = 8), suggesting that 1,25-dihydroxyvitamin D3 is able to promote osteogenic differentiation of normal ligament cells. Among the Japanese, sensitivity to vitamin D has been reported to vary between the alleles of the VDR; i.e., bone mineral density (BMD) in patients without the B allele is increased by vitamin D treatment, whereas patients with the B allele do not show such an increase in BMD. The present investigation is a small preliminary study, but the findings suggest, for the first time, that the B allele of the VDR acts as an inhibitor in the pathogenesis of human male OSL.

Key words ossification · ligament · vitamin D · vitamin D receptor · polymorphism

Introduction

Ossification of spinal ligaments (OSL) is a common form of human myelopathy characterized by heterotopic bone formation in the spinal ligaments that are normally composed of fibrous tissues. Among the Japanese population over 30 years of age, OSL has a prevalence of less than 5%, and predominantly occurs in men [1]. Genetic factors have been strongly implied in the etiology of OSL by epidemiological studies [2], and by association studies with alleles within the HLA region [3], although the genetic factors responsible have not been fully elucidated. Recently, nucleotide pyrophosphatase (NPPS), which produces inorganic pyrophosphate, an inhibitor of calcification, was reported to be genetically associated with human OSL [4]. We also reported that leptin is genetically involved in the pathogenesis of OSL in female patients, but not in male patients [5]. Indeed, Zucker fatty (fa/fa) rats with an aberration of the leptin receptor gene develop OSL [6], and a recent study has revealed that leptin is an inhibitor of bone formation [7]. It is well known that patients with X-linked hypophosphatemic vitamin D-resistant osteomalacia often develop OSL [8,9]. Although the mechanism is unknown, the OSL seen in this disease suggests the possible involvement of vitamin D in the etiology of OSL. Genetic factors in the development of osteoporosis have also been implied, and it has been reported that an allele defined by the BsmI polymorphism in the gene encoding the vitamin D receptor (VDR) is associated with bone mineral density (BMD) [10,11].

In the present preliminary study, we investigated the possible involvement of VDR gene polymorphism in male patients with OSL.
Materials and methods

Patients and samples

Blood samples for the analysis of VDR gene polymorphism were obtained from 27 male patients with myelopathy due to obvious OSL who underwent spinal surgery (mean age 62 years; range 35–84 years) and 97 age-matched male control subjects without symptoms of OSL (mean age 63 years; range 27–81 years). The diagnosis of OSL was made on the basis of clinical symptoms and radiologic examinations using tomography and computed tomography of the spine. Yellow ligaments of the lumbar spine for cell culture were also collected at spinal surgery [12] from another eight male patients without OSL (mean age 56 years; range 43–65 years). All subjects were unrelated Japanese, and were excluded from the study if they had diseases known to affect bone metabolism. The study was approved by the Institutional Review Board at Yamaguchi University Hospital, and all patients gave informed consent to participate.

Analysis of VDR gene polymorphism

The VDR genotype defined by the BsmI polymorphism was analyzed as reported previously [13]. DNA was extracted from leukocytes using standard methods, and the VDR gene was amplified by polymerase chain reaction. On the basis of whether the VDR gene was cleaved by the restriction enzyme BsmI, the VDR polymorphism was classified as B (not cleaved by BsmI) or b (cleaved by BsmI). Thus, subjects were divided into three groups: BB, Bb, or bb.

1,25-Dihydroxyvitamin D₃ stimulation of alkaline phosphatase activity in ligament cells

Yellow ligaments were collected en bloc aseptically. The fresh ligament was placed in α-minimum essential medium (α-MEM) after the surrounding tissues were completely removed. Ligament cells were obtained using methods reported previously [5]. Briefly, the ligament was cut into pieces in α-MEM, washed with α-MEM, placed in a 300-cm² flask containing α-MEM with 20% heat-inactivated fetal bovine serum (FBS), penicillin (100 units/ml) and streptomycin (100µg/ml), and then incubated at 37°C in a humidified atmosphere of 95% air and 5% CO₂. Explants were cultured for 14 days, with the medium changed on the 7th day, and outgrowth cells were detached by 0.05% trypsin and 0.53 mM EDTA for 5 min at 37°C. Collected cells were seeded in 12-well plates containing α-MEM with 10% heat-inactivated FBS, penicillin (100 units/ml) and streptomycin (100µg/ml) at a density of 5 × 10³ cells/cm². Then, cells were stimulated with 1,25-dihydroxyvitamin D₃ at concentrations of 0, 10⁻¹⁰, 10⁻⁹, or 10⁻⁸ M, and incubated for 9 days. The medium was changed once every 3 days. Alkaline phosphatase (ALP) activity, a parameter of osteogenic differentiation, was measured in the cell layer and standardized by referring to the protein content, as described previously [14,15]. Data are presented as the mean of four wells in each sample. 1,25-Dihydroxyvitamin D₃ was purchased from Solvay Pharmaceuticals (Weesp, The Netherlands), and dissolved in ethanol. The final concentration of ethanol in the medium was 0.01%.

Statistical analysis

The χ²-test was performed to examine the allele frequency of the VDR, and paired Student's t-test was performed for ALP activity, with significance of a two-sided test set at P < 0.05.

Results

With regard to the VDR genotype, of the patients with OSL (n = 27), none had the BB genotype (0%), one had the Bb genotype (4%), and 26 had the bb genotype (96%). In the control group (n = 97), three had the BB genotype (3%), 18 had the Bb genotype (19%), and 76 had the bb genotype (78%). As a result, the B allele frequency in patients with OSL (2%) was significantly lower than in controls (12%; P < 0.05; Fig. 1).

In ligament cells, 1,25-dihydroxyvitamin D₃, at concentrations of 10⁻¹⁰ (P < 0.05) and 10⁻⁸ M (P < 0.01), significantly increased ALP activity (Fig. 2). The effect was dose dependent.

Discussion

This is the first report showing the possible involvement of VDR gene polymorphism with the occurrence of human male OSL. In the present preliminary study, the B allele frequency in patients with OSL (2%) was markedly lower than in controls (12%). This result agrees with a finding that patients with OSL have a higher BMD than control subjects [16] on the basis of the relationship between the VDR alleles and BMD among Japanese [11,17]. A radiographic study of OSL was undertaken worldwide, and the incidence of OSL was found to be much higher in Japanese people than in Caucasians [1]. In fact, it was reported that the B allele frequency of the VDR in Japanese people (12%) was much lower than in Caucasians (41%) [17].

1,25-Dihydroxyvitamin D₃, at concentrations of 10⁻⁹ and 10⁻⁸ M, significantly increased ALP activity of liga-