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The Quantification of Vitamin D Receptors in Coronary Arteries and Their Association with Atherosclerosis

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Abstract

Objective—The activated vitamin D receptor (VDR) may have an important role in vascular health. The objective of this study was to determine whether there is an association between the expression of VDRs in coronary arteries and the extent of diet-induced atherosclerosis.

Methods—Utilizing a cohort of 39 postmenopausal female cynomolgus monkeys with varying stages of atherosclerosis, histologic sections of the left anterior descending artery (LAD) were analyzed for plaque cross-sectional area, plaque thickness, and VDR quantity using immunohistochemical H-score analysis. The quantities of VDRs were analyzed as a continuous variable and were divided at the median intimal H-score into high vs. low groupings.

Results—In the LAD, a significant negative correlation was observed between the quantity of VDR and plaque size (both cross-sectional area [$p < 0.001$] and plaque thickness [$p < 0.001$]). Monkeys in the low VDR group had a significantly greater cross-sectional plaque area (1.2 mm²) and greater plaque thickness (0.3 mm) than those in the high VDR group (0.4 mm², $p = 0.005$; 0.1 mm, $p = 0.003$, respectively).

Conclusions—Lower concentrations of VDRs in a main coronary artery were associated with greater atherosclerotic plaque size in postmenopausal female monkeys. Given that coronary artery

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atherosclerosis is a major cause of coronary heart disease in postmenopausal women, further research to ascertain the relationship between VDRs and atherosclerosis is warranted.

Keywords

Vitamin D Receptors; Coronary Artery Atherosclerosis; Coronary Heart Disease; Menopause; Translational Research

Introduction

Vitamin D (25-hydroxyvitamin D) plays an essential role in the prevention of osteoporosis and rickets; however, recent data suggest that extra skeletal benefits of 25-hydroxyvitamin D may exist, including the prevention of some cancers, autoimmune diseases, and coronary heart disease (CHD) [1]. In particular, there is increasing evidence linking a deficiency in 25-hydroxyvitamin D to coronary artery atherosclerosis [2-4]. The results of one study showed an inverse relationship between low plasma 25-hydroxyvitamin D concentrations and first-time cardiovascular events [4]. Low 25-hydroxyvitamin D has also been associated with a number of CHD risk factors, including obesity [5], hypercholesterolemia [6-8], diabetes mellitus [9], and hypertension [10]. The results of other studies have indicated a higher incidence of CHD and hypertension in higher latitudes and lower altitudes suggesting those with lower exposure to ultraviolet-B (UVB) radiation, a major source of 25-hydroxyvitamin D, may have a higher risk of heart disease [11,12].

Among women, CHD accounts for 420,000 deaths per year and is the leading cause of mortality [13]. In fact, more women than men die from CHD in the United States every year [13]. Therefore, the identification of risk factors and potentially treatable conditions, has tremendous public health implications.

The vitamin D receptor (VDR) is an intracellular receptor which binds 1,25-dihydroxyvitamin D, the active form of vitamin D [14]. The VDR has been identified in most human tissues (including the heart, brain, kidney, and muscle [15-21]) as well as in sheep arteries (endothelium, intima, and media [22,23]). The widespread distribution of VDR implies a diverse role for the receptor, beyond regulation of phosphorus and calcium plasma concentrations [20,21]. VDR activation allows targeted gene transcription [24] and has many important functions related to cardiovascular health which may promote the inhibition of atherosclerosis [25]. The most commonly cited mechanisms include inhibition of vascular smooth muscle cell proliferation [23], down-regulation of the renin-angiotensin-aldosterone system [26], inhibition of pro-inflammatory cytokines [25,27], regulation of matrix metalloproteinase-9 [28,29], and up-regulation of anti-inflammatory cytokines [30]. Furthermore, VDR-knockout mice have increased blood pressure, higher plasma angiotensin, increased renin, and more prevalent cardiac hypertrophy than wild-type mice [26,31-32].

Despite an extensive search, we were unable to find reports correlating the quantity of VDRs with coronary artery atherosclerosis. Although the results of previous studies indicate that higher concentrations of 1,25-dihydroxyvitamin D result in increased transcription and/or quantity of the VDR [33,34], our understanding of VDR expression in tissue, and the clinical ramifications relating to the coronary arteries, remains incomplete. For example, expression of the vitamin D receptor is reported to be increased in the cardiomyocyte heart, which subsequently may control hypertrophy through a counter-regulatory mechanism [15]. Therefore, the objective of this study was to quantify the expression of 25-hydroxyvitamin D receptors in histologic sections of the left anterior descending (LAD) coronary artery and evaluate the relationship between VDR number and extent of coronary artery

atherosclerosis, utilizing a cohort of surgically postmenopausal cynomolgus monkeys fed a moderately atherogenic diet.

Methods

Monkeys and diets

A cohort of 39 female cynomolgus monkeys (*Macaca fascicularis*), imported as adults from Indonesia (Institute Pertanian Bogor), was utilized for this study. After arriving at the Wake Forest University Primate Center, the monkeys were housed indoors with no UV exposure for 3 months prior to the beginning of the study. Adult status was confirmed by dentition and evidence of epiphyseal closure. Throughout the entire study, all 39 monkeys received moderately atherogenic diets with a women's equivalent of 1,000 IU/day of 25-hydroxyvitamin D₃ and 1,200 mg/day of calcium. All animals were housed indoors without UV exposure during the entire length of the study. During the premenopausal portion of the study, monkeys were fed either a diet containing casein/lactalbumin (C/L) or a soy protein isolate (soy) for 32 months. At this point, the monkeys underwent surgical menopause (bilateral oophorectomy). During the menopausal portion of the study, the monkeys were re-randomized to receive either the C/L or soy diet for 32 additional months. Therefore, a total of 10 received C/L-soy, 10 received C/L-C/L, 9 received soy-C/L, and 10 received soy-soy. Both diets comprised 19% of calories from protein, 35% of calories from fat, and 46% of calories from carbohydrate, with 0.28 mg of cholesterol/cal. The soy protein isolate contained 1.88 mg aglycone isoflavones/g protein (SUPRO® SOY Isolated Soy Protein, Solae St. Louis, MO).

An adiposity index for the monkeys was derived by measuring the body weight divided by the trunk length (squared), and has a normal range of 35 to 55 kg/m². Trunk length is measured from the sternal notch to the pubic symphysis.

Atherosclerosis Measurements

At necropsy (at the end of the menopausal phase of the study), the heart was perfusion-fixed at physiologic pressure (100mm Hg) in 4% paraformaldehyde. After 24 hours, the heart was transferred into 70% ethanol. Each main coronary artery, including the left anterior descending (LAD) and left circumflex (LCX) were removed and cut into 5 x 5mm blocks and embedded in paraffin. Deparaffinized sections (5 µm) were then obtained and stained with Verhoeff and Van Gieson stain. Cross sectional area (mm²) and plaque thickness (mm) of the LAD and LCX biopsy sections were determined by computer-assisted histomorphometry using Image Pro Plus software (Media Cybernetics, Inc., Silver Springs, MD). Measurements were made by an experienced technician blinded to the treatment group using a well-established protocol [35].

Immunolocalization of the VDR and H-score analysis

For the quantification of VDR, to eliminate the potential of intra or interobserver variation and interpretation bias, one consistent and blinded observer was used for all measurements. In order to assure validity and reproducibility, however, the following steps were taken before quantification and observations were carried out. The designated observer met with other trained observers to co-analyze specimens assuring consistent and reproducible results.

The quantity of coronary artery VDRs was subsequently determined for the LAD by determining the intimal H-Score. Artery blocks were cut to 5 µm sections, which were deparaffinized and immunohistochemically stained for the VDR. Sections were treated with rat anti-Vitamin D Receptor Monoclonal Antibody (Thermo Scientific, Rockford, IL) and then treated with biotinylated goat anti-rat (Serotec, Raleigh, NC) with the enzyme

conjugate streptavidin-alkaline phosphatase (BioGenex, San Ramon, CA) and enzyme substrate vector red (Vector Laboratories, Burlingame, CA). For counting, only a hematoxylin counterstain was used; the internal elastic lamina was visible as a refractile line. For demonstration of the internal elastic lamina for photomicroscopy, slides were further stained for elastin using a modified Weigert resorcin-fuchsin method [36], hematoxylin, and metanil yellow.

All cells in the intima, media, and adventitia were counted using a light microscope at 400X magnification and a key counter. The adventitia was defined as a half 400X field width from the outermost medial smooth muscle layer. An H-Score was calculated using a method described by Siboni et al. [37]. All cells were assigned a grade of 0, 1, 2, or 3, where a grade 0 indicated a nucleus with no red stain, a grade 1 indicated a slightly red stained nucleus, a grade 2 indicated a moderately stained red nucleus, and a grade 3 indicated a dark and diffusely stained red nucleus. The intimal H-score was determined from calculating 3 times the percentage of grade 3 cells plus 2 times percentage of grade 2 cells plus 1 times the percentage of grade 1 cells plus 0 times the percentage of grade 0 cells in the intima. This summative score, with a possible minimum of 0 and a possible maximum of 300, was referred to as the intimal H-Score.

Statistical analysis

Descriptive statistics including median, means, and standard deviations (SDs) were used to describe the VDR data, cross-sectional plaque area, and plaque thickness. VDR quantity was analyzed as a continuous variable and subsequently was dichotomized into higher and lower concentrations using the median intimal H-score of 61. After evaluating the data for normality, inferential statistics consisted of Student's *t* test to compare plaque thickness, cross-sectional area, and VDR group (high vs. low). For the comparison of plaque thickness, cross-sectional area and VDR intimal H-score for the entire cohort (n=39), Pearson's correlation coefficient was used to generate *r* values for continuous variables. Correlation between variables and high and low VDR groups were analyzed using Spearman ranked correlation coefficient to generate Spearman's rho values.

All analyses were conducted using an a priori α level of 0.05 such that results yielding $p < 0.05$ were deemed statistically significant. SPSS version 17.0 (SPSS Inc., Chicago, IL) was used for all analyses.

Approval

All procedures involving animals in this study were conducted in compliance with state and federal laws, standards of the US Department of Health and Human Services, and guidelines established by the Wake Forest University Institutional Animal Care and Use Committee, where the animals were housed.

Results

Among the 39 monkeys, the estimated age (mean $\pm$ SD) at necropsy was 21.6 ± 3.0 years with a range of 14.2 to 26.3 years. The mean body weight at this time was 3.6 ± 0.9 kg and the mean adiposity index was 49.8 ± 11.3 kg/m². Comparing the demographics between the monkeys with higher, vs. lower, quantities of VDRs, the median intimal H-Score was used. There were no significant differences in age, body weight, or adiposity index between the two groups.

Using the intimal H-score as a continuous variable, we were able to determine the association between both LAD plaque thickness and cross-sectional area, and the expression of VDRs. A significant negative correlation was observed between the quantity of VDR and

LAD plaque size (both cross-sectional area [$p<0.001$, figure 1] and plaque thickness [$p<0.001$]). After controlling for differences in plasma lipid concentrations (the total plasma cholesterol concentrations (TPC) and the TPC to high-density lipoprotein cholesterol ratio (TPC:HDL-C), the significant negative correlation remained.

Monkeys with an intimal H-score at or above the median of 61 had a significantly smaller LAD plaque thickness than did those with an intimal H-score below the median value (0.13 ± 0.14 mm vs. 0.33 ± 0.22 mm, respectively; $p=0.003$, figure 2). The LAD cross-sectional area was significantly lower in the group with higher intimal H-scores (0.39 ± 0.52 mm²) compared with the group with lower intimal H-scores (1.16 ± 0.95 mm²; $p=0.005$, figure 2).

Because the expression of VDR in the LAD reflects the likely expression in the other main epicardial coronary arteries, we extended our analyses to include the left circumflex coronary artery (LCX). Monkeys with a LAD intimal H-score at or above the median had a significantly smaller LCX plaque thickness than did those with a LAD intimal H-score below the median value (0.15 ± 0.12 mm vs. 0.35 ± 0.23 mm, respectively; $p=0.002$). The LCX cross-sectional area was significantly lower in the group with higher LAD intimal H-scores (0.40 ± 0.47 mm²) compared with the group with lower LAD intimal H-scores (1.09 ± 0.84 mm²; $p=0.003$).

Monkeys with higher LAD intimal H-scores had a more beneficial postmenopausal plasma lipid profiles than their counterparts with LAD intimal H-scores below the median value. TPC was significantly lower (275.3 ± 99.9 mg/dL vs. 353.1 ± 94.3 mg/dL, resp.; $p=0.019$). Similarly, HDL-C concentrations were significantly higher in those with greater, vs. lesser, LAD intimal VDR expression (63.4 ± 24.8 mg/dL vs. 46.7 ± 18.6 mg/dL, resp.; $p=0.026$). There was, however, no significant difference between groups with mean triglyceride concentrations (43.7 ± 33.8 vs. 35.8 ± 23.3 , resp.; $p=0.415$), see table 1.

Discussion

The results of this study demonstrate an association between low concentrations of VDRs in the LAD and extent of atherosclerosis. Postmenopausal cynomolgus monkeys with higher VDR concentrations had significantly smaller atherosclerotic plaque areas (Figure 1) and plaque thickness. These findings suggest that coronary artery plaque burden, the major cause of coronary heart disease, may be linked to decreased concentrations of VDRs in the monkey model.

Whether the progression of coronary artery atherosclerosis leads to a down-regulation of coronary artery VDRs or whether a low expression of coronary VDRs results in a greater likelihood of coronary artery atherosclerosis is not known. If low numbers of coronary VDRs enhance atherosclerotic plaque development, it would suggest that the presence of VDRs, and hence their ability to allow 25-hydroxyvitamin D to work locally, may play a substantial role in coronary atheroprotection. While it is plausible that the presence of coronary artery atherosclerosis results in a local loss of VDR receptors, there is evidence to suggest the reverse could be true. In our study, for example, monkeys in the lower grouping of VDR quantity had significantly greater TPC and lower HDL-C concentrations, both well-recognized risk factors for developing CHD¹³. Our analysis of VDR data incorporating systemic effects on plasma concentrations of 25-hydroxyvitamin D₃ also showed a significant relationship [38]. It is difficult to propose a mechanism whereby low coronary artery VDR expression results in alterations in plasma TPC or HDL-C concentrations. The reverse situation, however, in which a more atherogenic plasma lipid profile results in decreased arterial VDRs is plausible. Some other potential reasons for reduced arterial VDRs include genetic predisposition and systemic toxins.

The potential relationship between 25-hydroxyvitamin D and the extent of atherosclerosis is not unlike the reported relationship, and “timing hypothesis”, suggested between hormonal therapy (HT) / estrogen therapy (ET) and atherosclerosis [39,40]. It is even possible that estrogen and 25-hydroxyvitamin D may have synergistic effects in the artery since estrogen can influence 25-hydroxyvitamin D metabolism and *vice versa* [41,42]. The results of our study closely parallel the results of previous studies of estrogen receptor quantity in the coronary arteries and its association with atherosclerosis [43,44]. The results of one study found that in women, coronary arteries with diminished estrogen receptors revealed greater atherosclerosis compared to arteries with more abundant estrogen receptors, which showed significantly less atherosclerosis [43]. Additionally, methylation of the vascular estrogen receptor has been associated with increased atherosclerosis [44]. Finally, the results of another study suggest that estrogen may up-regulate the VDR [45], a potential explanation for cardiovascular benefit in younger, healthier, women on HT or conversely why CHD dramatically increases during the postmenopausal years [46,47].

A limitation to this study is the cross-sectional nature of the data. We cannot conclude with certainty the cause and effect relationship between VDR quantity and atherosclerosis progression. There are several strengths of this study that deserve mention, including the ability to control dietary and environmental vitamin D exposure along with the collection of coronary arteries from a well-established model of atherosclerosis development in postmenopausal females [40]. In fact, the ability to control and analyze diet as part of a possible explanation for the findings presented adds to the strength of the study. Diet did not have a significant role in explaining these outcomes.

In conclusion, the results of our study suggest a role for the VDR in the progression of atherosclerosis, although a casual relationship cannot yet be established. Further research to determine the effect of Vitamin D deficiency and supplementation on progression of atherosclerosis in a prospective, longitudinal manner is required.

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Figure 1.

For the 39 female cynomolgus monkeys in this study, this graph depicts the atherosclerotic plaque size (as a function of the cross-sectional area of the left anterior descending [LAD] artery) in relation to the expression of vitamin D receptors (VDRs) in the coronary arteries. As the expression of VDRs decreased, the LAD atherosclerotic plaque size significantly increased.

Figure 2.

For the 39 female cynomolgus monkeys in this study, this graph depicts the plaque thickness (or maximal intimal thickness) of the left anterior descending (LAD) artery [left side of the graph] and the atherosclerotic plaque area of the LAD artery [right side of the graph] in those with a lower vs. a higher vitamin D receptor (VDR) expression. The LAD VDR quantity was dichotomized into higher and lower concentrations using the median H-score of 61.

Table 1

Comparison of plasma lipid profiles between higher and lower intimal H-scores

Lipid parameter (units)	Value (mean $\pm$ SD) for higher intimal H-score*	Value (mean $\pm$ SD) for lower intimal H-score*	P value of difference (t-test)**
TPC (mg/dL)	275.3 $\pm$ 99.9	353.1 $\pm$ 94.3	0.019
HDL-C (mg/dL)	63.4 $\pm$ 24.8	46.7 $\pm$ 18.6	0.026
TG (mg/dL)	43.7 $\pm$ 33.8	35.8 $\pm$ 23.3	0.415

* "higher intimal H-score" refers to those $\geq$ the median; "lower intimal H-score" refers to those < the median.

** values shown in **bold** are statistically significant at $p < 0.05$.