Vitamin D deficiency: Correlation to interleukin-17, interleukin-23 and PⅢNP in hepatitis C virus genotype 4

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Author contributions: Mohamed WA and Amin HH contributed to the acquisition and analysis of data; Schaalan MF and Mohamed WA contributed to the interpretation of data and drafting the article; Schaalan MF revised the manuscript critically for important intellectual content and approved the final version to be published; all authors contributed equally to the conception and design of the study.

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Received: December 13, 2011 Revised: April 19, 2012 Accepted: April 27, 2012 Published online: July 28, 2012

Abstract

AIM: To assess vitamin D (Vit D) abnormalities in hepatitis C infected patients and their relationship with interleukin (IL)-17, IL-23 and N-terminal propeptide of type Ⅲ pro-collagen (PⅢNP) as immune response mediators.

METHODS: The study was conducted on 50 Egyptian patients (36 male, 14 female) with hepatitis C virus (HCV) infection, who visited the Hepatology Outpatient Clinic in the Endemic Disease Hospital at Cairo University. Patients were compared with 25 age- and sex-matched healthy individuals. Inclusion criteria were based on a history of liver disease with HCV genotype 4 (HCV-4) infection (as new patients or under follow-up). Based on ultrasonography, patients were classified into four subgroups; 14 with bright hepatomegaly; 11 with perihepatic fibrosis; 11 with hepatic cirrhosis; and 14 with cirrhosis and hepatocellular carcinoma (HCC).

Total Vit D (i.e., 25-OH-Vit D) and active Vit D [i.e., 1,25-(OH)2-Vit D] assays were carried out using commercial kits. IL-17, IL-23 and PⅢNP levels were assayed using enzyme linked immunosorbent assay kits, while HCV virus was measured by quantitative and qualitative polymerase chain reaction.

RESULTS: Levels of Vit D and its active form were significantly lower in advanced liver disease (hepatic cirrhosis and/or carcinoma) patients, compared to those with bright hepatomegaly and perihepatic fibrosis. IL-17, IL-23 and PⅢNP levels were markedly increased in HCV patients and correlated with the progression of hepatic damage. The decrease in Vit D and active Vit D was concomitant with an increase in viral load, as well as levels of IL-17, IL-23 and PⅢNP among all subgroups of HCV-infected patients, compared to normal healthy controls. A significant negative correlation was evident between active Vit D and each of IL-17, IL-23 and PⅢNP (r = -0.679, -0.801 and -0.920 at P < 0.001, respectively). HCV-infected men and women showed no differences with respect to Vit D levels. The viral load was negatively correlated with Vit D and active Vit D (r = -0.084 and -0.846 at P < 0.001, respectively), and positively correlated with IL-17, IL-23 and PⅢNP (r = 0.951, 0.922 and 0.94 at P < 0.001, respectively). Whether the deficiency in Vit D was related to HCV-induced chronic liver disease or was a predisposing factor for a higher viral load remains to be elucidated.

CONCLUSION: The negative correlations between Vit D and IL-17, IL-23 and PⅢNP highlight their involvement in the immune response in patients with HCV-related liver diseases in Egypt.

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Key words: Vitamin D; Interleukin-17; Interleukin-23; N-terminal propeptide of type Ⅲ pro-collagen; Hepatitis genotype 4
INTRODUCTION

Hepatitis C virus genotype 4 (HCV-4) is the most common variant of hepatitis C virus (HCV) in the Middle East and Africa, particularly Egypt. This region has the highest prevalence of HCV worldwide, with > 90% of infections due to HCV-4, which is considered a major cause of chronic hepatitis, liver cirrhosis, hepatocellular carcinoma (HCC), and liver transplantation in the country[3]. HCV-4 has recently spread beyond its strongholds in Africa and the Middle East to several western countries, particularly in Europe, due to variations in population structure, immigration and routes of transmission. However, the features of this genotype and management strategies for patients infected with this genotype are not well developed[3].

HCV is remarkably efficient at establishing persistent infections. This suggests that HCV has evolved one or more strategies to evade the host immune response, among which are effects upon T-lymphocyte responses, including interferon (IFN)-γ production; documented by the severely suppressed T-lymphocyte responses in patients with chronic HCV infections[9].

Vitamin D (Vit D) is a critical regulator of immunity, playing a role in both innate and cell-mediated immune responses[8,9]. Vit D suppresses production of T-helper (Th)-1 lymphocyte type cytokines, such as IFN-γ and interleukin (IL)-2, and consequently leads to an enhanced production of Th2 cytokines, such as IL-4 and IL-5, thereby promoting humoral immune responses. Vit D also endorses innate immunity by directly inducing gene expression of antimicrobial peptides, such as cathelicidin and β-defensin 2, in various human cell types[8,9]. Vit D deficiency has been shown to associate several immune-mediated diseases, as well as to increase susceptibility to both infections and cancer. Specifically, a 25(OH)-Vit D concentration < 50 nmol/L (i.e., 20 ng/mL) is accepted as a marker of deficiency, whereas a concentration of 51-74 nmol/L (21-29 ng/mL) indicates insufficiency[8,9].

Chronic hepatic cirrhotic patients with HCV genotype 1 have low serum 25(OH)-Vit D levels, and a low Vit D status is linked to severe fibrosis and a low sustained virological response (SVR) during IFN-α-based therapy[8,11]. Moreover, there is interesting preliminary data that indicate that 1,25-(OH)2Vit D suppresses Th1 driven cytokine responses and the differentiation and maturation of B lymphocytes, while it induces formation/activation of T-regulatory lymphocytes, stimulates IL-4 production (Th2), and enhances natural-killer-T-cell functions[5-8].

It has also been shown that treatment with Vit D receptor (VDR) agonists inhibits T-lymphocyte production of IL-17, which is a potent mediator of delayed-type reactions. It achieves this effect (in a manner similar to IFNγ) by elevating chemokine production in various tissues that, in turn, leads to recruitment of monocytes and neutrophils to the site of inflammation. Furthermore, IL-17 acts synergistically with tumor necrosis factor (TNF)-α and IL-1β to stimulate immune functions, and its production is sustained by IL-23, an IL-12 family member, the latter of which is strongly inhibited by VDR agonists[8]. IL-23, in conjunction with IL-6 and transforming growth factor (TGF)-β, also stimulate the differentiation of Th17 cells with the subsequent production of IL-17[6].

The aim of the work was to assess Vit D status in HCV-4-infected patients and its relationship to levels of IL-17 and IL-23, as well as the N-terminal propeptide of type III pro-collagen (PⅢNP), the direct serologic marker of collagen turnover, as immuno-inflammatory mediators.

MATERIALS AND METHODS

Subjects

Prior to initiation, this study received approval by the Ethical Committee of the Faculty of Medicine, Cairo University. The study recruited 50 patients with HCV-related chronic liver disease (minimum duration of 7 years; group I) who visited the Hepatology Outpatient Clinic in the Endemic Disease Hospital at Cairo University. Inclusion criteria were based on a history of liver disease with HCV-4 infection (as new patients or under follow-up). Patients with hepatitis B virus (HBV) or coinfection with HBV and human immunodeficiency virus were excluded. All included patients underwent tests for liver function and abdominal ultrasonography, and were tested for the presence of HCV antibodies. When fulfilled, the investigated patients included 36 men and 14 women, ranging in age from 30 to 55 years (mean age = 42.5 years). Twenty-five age- and sex-matched healthy individuals were then recruited as a control group (group II). The controls had normal liver functions and abdominal ultrasonography and negligible HCV antibody levels. Informed consent was obtained from the patients and controls regarding all the procedures. All patients were subjected to a thorough history taking.

Based on ultrasonography results, patients were classified into four subgroups: 14 with bright hepatomegaly; 11 with perihepatic fibrosis; 11 with hepatic cirrhosis; and 14 with cirrhosis and HCC. After subclassification, venous blood samples (5 mL) were obtained (after overnight fast-
Table 1 Levels of vitamin D, its active form, interleukin-17, interleukin-23, N-terminal propeptide of type III procollagen and viral load in the four subgroups of hepatitis C virus-infected patients

| Item                  | Group (a) | Group (b) | Group (c) | Group (d) | Group (e) |
|-----------------------|-----------|-----------|-----------|-----------|-----------|
| Vitamin D (ng/mL)     | 19.80 ± 3.33 | 19.40 ± 3.52 | 10.90 ± 3.74 | 9.70 ± 3.88 | 39.70 ± 10.80 |
| IL-17 (ng/mL)         | 20.60 ± 3.50 | 21.00 ± 3.44 | 11.70 ± 2.52 | 11.70 ± 2.52 | 41.90 ± 7.90 |
| IL-23 (ng/mL)         | 7.60 ± 2.66 | 5.10 ± 2.44 | 115.90 ± 38.70 | 150.30 ± 46.80 | 1.20 ± 0.40 |
| Viral load (IU/mL)    | 76.80 ± 14.51 | 51.20 ± 14.60 | 259.30 ± 49.4 | 225.90 ± 42.10 | 6.70 ± 2.17 |
| PⅢNP (μg/L)           | 66.30 ± 23.55 | 42.40 ± 9.66 | 165.10 ± 31.40 | 231.10 ± 44.60 | 0 ± 0 |
| Values shown are means ± SD of data in four subgroups of hepatitis C virus-infected patients and normal controls. a, b, d, h means within lines with no common superscripts differ significantly. As compared with the group (a), *group (b), group (c), *control (group e) (one-way analysis of variance followed by Tukey-Kramer test), P < 0.01. HCC: Hepatocellular carcinoma; IL: Interleukin.

Assessment of Vit D, active Vit D, IL-17, IL-23 and PⅢNP levels

Total Vit D (i.e., 25-OH-Vit D) assay was carried out using a commercial solid phase radioimmunoassay kit (Medgenix Diagnostics SA Zoning Industrial, Fleurus, Belgium) according to the method of Mawer [18]. The active Vit D (i.e., 1,25(OH)2-Vit D) assay was carried out according to Hossili [19] using a commercial kit from InStar Corporation (Stillwater, MN, United States). IL-17, IL-23 and PⅢNP levels were assessed using enzyme linked immunosorbent assay kits obtained from Biosource Europe SA (Nivelles, Belgium). The sensitivity of the 25-OH-Vit D, 1,25(OH)2-Vit D, IL-17, IL-23 and PⅢNP kits were 2.4 ng/mL, 5.5 pg/mL, 10 pg/mL, 5 pg/mL, and 10 pg/mL, respectively.

Assessment of HCV levels

Quantitative reverse transcription polymerase chain reaction (RT-PCR) for HCV was done using TaqMan technology according to the method of Scott and Gretch [20], and only HCV-4-infected patients were included in the study. Typically, an RT-PCR has a limit of quantification (LOQ) of 25 IU/mL and a limit of detection (LOD) of 10-15 IU/mL; in the assays used here for HCV-RNA testing, the LOQ was 24 IU/mL and the LOD 10-15 IU/mL.

For genotyping, HCV type-specific primers designed by Okamoto et al. [26] were utilized. Assessments of genotypes burdens required three steps: (1) RNA virus was extracted from patient samples using a TriPure Method (Roche, Mannheim, Germany); (2) isolated RNA was converted to cDNA using random hexamers and Moloney Murine Leukemia Virus Reverse Transcriptase enzymes from Promega (Madison, WI, United States); and (3) the product cDNA was amplified using an allele-specific PCR method. The PCR program was set for 1 cycle at 96 °C for 6 min, then for 40 cycles at 95 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min, and a final extension cycle of 72 °C for 10 min. For each patient, two vials containing primer specific for virus types 1a/1b and for 2a and 3a were used. A positive control for each genotype (supplied by the kit manufacturer) was also run in parallel with each set of samples. In addition, HCV viral load was also determined using an Artus Real Art PCR Kit (Qiagen, Valencia, CA, United States). For both assays, a LightCycler® 480 real-time PCR System (Roche) was used. The slope of each reaction was between 3.2 and 3.4, and the error < 0.002. All results of the quantitative HCV analyses were expressed as IU/mL.

Statistical analysis

All data are expressed as mean ± SD. All analyses utilized SPSS for Windows version 15.0 (SPSS, Chicago, IL, United States). Analysis of variance was employed for comparisons of means of the different parameters, with Tukey-Kramer test as the post hoc test. P < 0.05 was accepted as statistically significant. Correlation analyses were done using Pearson's correlation.

RESULTS

Table 1 demonstrates significantly decreasing levels of Vit D and active Vit D [1,25(OH)2-Vit D] along with the progressive hepatic state induced by chronic HCV infection, ranging from bright hepatomegaly and perihepatic fibrosis to hepatic cirrhosis and further HCC. Levels of Vit D and active 1,25(OH)2-Vit D were significantly lower in advanced liver disease (i.e., hepatic cirrhosis and/or carcinoma), compared to bright hepatomegaly and perihepatic fibrosis. Only the IL-17, PⅢNP and the viral load of patients with HCC were significantly higher than in cirrhotic patients. The decrease in Vit D and active Vit D was concomitant with increases in viral load, as well as levels of IL-17, IL-23 and PⅢNP among all subgroups of HCV-infected patients, compared to normal healthy controls.

Vit D insufficiency (21-29 ng/mL) was detected in 14 (28%) HCV patients and three (12%) controls, while Vit
Vitamin D deficiency, defined as serum level < 20 ng/mL, was present in 36 (72%) of the HCV-infected patients and none of the controls. Lastly, Vit D deficiency was seen in all 25 cirrhotic patients and 10 (40%) of the non-cirrhotic HCV-infected patients.

The parameters studied in HCV-infected patients when classified into two subgroups according to sex are shown in Table 2. HCV-infected men and women showed no differences with respect to Vit D levels. There were significant correlations between different parameters in HCV-infected patients and controls. There was a significant negative correlation between Vit D and viral load, and active Vit D levels ($r = -0.846$ at $P < 0.001$), and positively correlated with IL-17, IL-23, and PⅢNP ($r = 0.951, 0.922$ and $0.94$ at $P < 0.001$). The significant negative correlations between active Vit D and IL-17 ($r = -0.679$), IL-23 ($r = -0.801$), and viral load ($r = -0.84$), are illustrated in Figure 1.

**DISCUSSION**

The liver plays a central role in Vit D metabolism, and its inadequacy is common in non-cholestatic chronic liver diseases and correlates with disease severity[21]. The current study showed a significant reduction of Vit D and its active metabolite in HCV-4-infected patients compared to healthy controls. HCV-infected patients were classified according to sonar finding into four groups with progressive hepatic states; bright hepatomegaly and perihepatic fibrosis to hepatic cirrhosis and further HCC. The reduction of the levels of Vit D and its active form was more prevalent and severe in cirrhotic patients (versus non-cirrhotic patients), and much lower in patients with HCC; these differences were each highly significant. This is consistent with previous studies in patients with HCV genotype 1, which showed that Vit D deficiency is universal (92%) among patients with chronic liver disease, and at least one-third of the patients have severe Vit D deficiency[21,22].

A significant negative correlation was reported between viral load and active Vit D levels in HCV-infected patients. Vit D is an important immune modulator and preliminary data have indicated associations between Vit D deficiency and failure to achieve SVR rates in HCV patients[24]. It has been reported that the reduced (25-OH Vit D) levels and CYPB27-1260 promoter polymorphism lead to reduced [1,25-(OH)2 Vit D] levels, and are associated with failure to achieve an SVR in patients infected with HCV genotypes 1, 2 or 3[14,25]. The patients in the present study with HCV-4 need further follow-up to confirm the effect of Vit D deficiency upon
their responses to treatment.

Furthermore, the present study showed that IL-17 and IL-23 were markedly increased in HCV-infected patients in comparison to controls. The difference in viral load among these groups may explain, in part, the differences noted in levels of inflammatory cytokines in the patients in the current study. Regulation of Th1 and Th17 responses in HCV-infected individuals has previously been studied[29], and it has been reported that TGF-β and IL-6 promote differentiation of naïve murine CD4+ T lymphocytes into IL-17-secreting Th17 lymphocytes. In addition, it has been reported that other innate cytokines, including IL-1, II-23, TNF-α and IL-21, in different combinations or with TGF-β, are also involved in the stabilization of the Th17 phenotype[29]. A significant negative correlation between Vit D and both IL-17 and IL-23 was a prominent finding in the current investigation. Previous studies in mice have shown that Vit D is a strong inhibitor of Th17 polarization and Th17 cytokine expression by splenic CD4+ T lymphocytes. Furthermore, Th17 differentiation from naïve T lymphocytes is affected by Vit D. These data imply a regulatory effect on Th17 cells by Vit D, through the reduction of retinoic acid-related orphan receptor (ROR)γt expression[22]. The effect of Vit D on the behavior of Th17 cells has been investigated in different diseases and Vit D suppresses the expression of IL-17 and II-23[22-28], as documented in the current study.

We reported a positive correlation between IL-17 and IL-23 and viral load; a finding that supports our hypothesis regarding a link between Vit D and both IL-17 and IL-23 in immunoregulation of HCV-4-related chronic liver disease, and may explain how Vit D deficiency plays a role in increasing liver fibrosis. Our results also revealed no significant differences between HCV-infected men and women with respect to Vit D status. In contrast, Arteh et al[30] have reported that African American women with chronic liver disease are at higher risk of Vit D deficiency.

During the process of liver fibrosis, type III procollagen is converted to type III collagen by cleavage of its amino terminal and carboxy terminal propeptides. Serum levels of IIINP, which are direct serologic markers of collagen turnover in liver fibrosis, are elevated in both acute and chronic liver diseases; and further reflect the histologic stage of hepatic fibrosis in various chronic liver diseases[31]. There was a significant increase (in comparison to controls) in the serum levels of IIINP in patients with all grades of hepatic disease; results that are in agreement with Walsh et al[32]. However, Panasiku et al[33] have reported a decrease in IIINP levels in cirrhotic patients in comparison to controls, and have not shown any inflammatory process in the cirrhosis, hence more studies are needed to resolve this point of controversy. The results of the current study also revealed a significant negative correlation between Vit D and IIINP levels, supporting a role for decreased Vit D in inflammation and fibrosis; a relationship that has not previously been investigated in patients with hepatic disease. Interestingly, Zehnder et al[34] have reported that reduction of the Vit D hormonal system in kidney diseases is associated with increased renal inflammation and fibrosis. These investigators have also reported a significant negative correlation between Vit D and IIINP levels. Logistic regression analysis with urinary IIINP, as a binary outcome, has shown that a 10-U increase in serum 1,25(OH)2-D or 25-OH-Vit D resulted in lower renal inflammation[35].

In conclusion, Vit D deficiency is prevalent in HCV-4-infected patients and the viral load is negatively correlated with Vit D status. In view of the role of Vit D in maintaining optimal immune function, Vit D status may be assessed and supplements may be considered to achieve an SVR during IFN-α-based therapy. The negative correlation between Vit D and IL-17, IL-23 and IIINP levels appears to highlight, at least in part, how these cytokines are involved with Vit D in immune responses in HCV-4-related liver disease, and could explain how Vit D deficiency plays a role in liver fibrosis.

ACKNOWLEDGMENTS

The authors extend special thanks to the workers at the Hepatology Clinic in the Endemic Disease Hospital, Cairo University for their help during this research.

COMMENTS

Background

Hepatitis C virus (HCV) infects primarily the hepatocytes, leads to the development of fibrosis or cirrhosis of the liver, and is a significant risk factor for the development of hepatocellular carcinoma (HCC). The cell-mediated immune response plays a central role in hepatocellular necrosis and in the immunopathogenic mechanisms involved in viral clearance and persistence in liver disease of viral etiology, such as HCV-related chronic liver disease. In this context, cytokines modulate the immune system and exert direct antiviral activity by cytotoxic and non-cytotoxic mechanisms. T-cell immunoregulatory cytokines influence the persistence of HCV chronic infection and the extent of liver damage.

Research frontiers

Vitamin (Vit D) abnormalities are well documented in patients with chronic liver disease, but the association of the degree of Vit D abnormality with the progressive hepatic necroinflammatory state has not been thoroughly investigated. Furthermore, the proinflammatory cytokine profile in patients infected with HCV genotype 4 (HCV-4) needs further study. The research hotspot is how to determine/assess the extent of Vit D abnormality in HCV-4-infected patients and determine its relationship with proinflammatory markers, namely, interleukin (IL)-17, IL-23, and the N-terminal propeptide of type III pro-collagen (IIINP) as immune response mediators.

Innovations and breakthroughs

A plethora of studies has investigated the association of Vit D abnormalities with individual liver diseases, including hepatitis B, alcoholic hepatitis, autoimmune hepatitis, and HCC. Nevertheless, the role of Vit D abnormalities in the progression of HCV to cirrhosis and then to HCC remains to be elucidated. To this end, this study sought to investigate the potential association between levels of Vit D and IL-17, IL-23 and exacerbation of hepatic damage in chronic HCV patients. The results showed that levels of Vit D and its active form were significantly lower in patients with advanced liver disease (hepatic cirrhosis and/or carcinoma), compared to those with white hepatomegaly and perihepatic fibrosis. IL-17, IL-23 and IIINP levels were markedly increased in HCV patients and correlated with progression of hepatic damage. The decrease in Vit D and its active form was concomitant with increases in viral load, as well as levels of IL-17, IL-23 and IIINP among all subgroups of HCV-infected patients, com-
pared to normal healthy controls. The negative correlation between Vit D and IL-17, IL-23 and P1NP may highlight, at least in part, how these cytokines are involved with Vit D in the immune response to HCV-4-related liver disease, and may explain how Vit D deficiency plays a role in the progression of liver fibrosis. In view of the role of Vit D in maintenance of immune function, its status may be assessed and supplements should be considered to achieve a sustained virological response during therapy.

Applications

The actual role of Vit D in the context of hepatic inflammatory process is still not fully elucidated. Given the major significance of the inflammatory response in mediating HCV clearance, as well as the anti-inflammatory actions displayed by Vit D in vitro, Vit D could have a positive influence on HCV infection. Further studies are needed to explain which stages of HCV infection require higher levels of Vit D and the mechanism of Vit D supplementation for these patients.

Terminology

Hepatitis C is a chronic liver infection that can be complicated by liver failure and liver cancer. In the liver, cytokines coordinate physiological and pathological processes such as liver growth and regeneration, inflammatory processes including viral liver disease, liver fibrosis and cirrhosis. T-cell immunoregulatory cytokines may play a key role in influencing the persistence of HCV infection and the extent of liver damage. IL-6 and transforming growth factor β (TGF-β) as the differentiation factors for Th17 cells, both cytokines together induce massive amounts of IL-17 from naïve T cells. Vit D has an important role in the treatment of different bacterial and viral infections; this vitamin is synthesized in the skin by absorption of ultraviolet light from the sun. The mechanism of action of this vitamin is unknown, but it may improve the activities of immune cells that are important in the eradication of HCV.

Peer review

This article reveals the importance of Vit D and/or its active form in HCV-4 infection, which is the most common form of hepatitis C in Egypt. The study also showed that this deficiency progresses with disease deterioration. This may indicate that Vit D supplements could be efficient in early stages of the disease.

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S-Editor Gou SX  L-Editor Kerr C  E-Editor Zhang DN