Vitamin D₃ Treatment Decreases Frequencies of CD16-Positive and TNF- α -Secreting Monocytes in Asthmatic Patients

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Key Words

Vitamin D₃ · Monocyte subsets · Innate immunity · Glucocorticoids · Asthma

Abstract

Background: Previously, we demonstrated that glucocorticoid (GC) treatment of asthmatic patients resulted in decreasing frequencies of monocyte subsets expressing CD16 and capable of releasing TNF- α . Here, we wished to analyze whether the active form of vitamin D, i.e. vitamin D₃, referred to as 1 α ,25-dihydroxyvitamin D₃ [1,25-(OH)₂D₃] can exert GC-like proapoptotic effects on CD16-positive monocytes and thus decrease the proinflammatory potential of these cells. Finally, we set out to investigate whether the addition of 1,25-(OH)₂D₃ would facilitate the use of lower doses of GC without decreasing their anti-inflammatory properties. **Methods:** Peripheral blood mononuclear cells collected from healthy individuals and asthmatic patients were cultured with 1,25-(OH)₂D₃ and/or varying doses of GC in the presence or absence of caspase inhibition. The cells were either directly stained for extracellular markers or prestimulated with lipopolysaccharide for the assessment of intracellular cytokine production and then analyzed by flow cytometry. **Results:** We found that 1,25-(OH)₂D₃ alone (and in combination

with GC) decreased the frequency of CD14++CD16+ and CD14+CD16++ monocytes from asthmatic patients and significantly diminished TNF- α production by the monocytes. With regard to the CD14+CD16++ subset, the monocyte-depleting effects of 1,25-(OH)₂D₃ were abrogated in the presence of pan-caspase inhibitor, suggesting a proapoptotic mechanism of 1,25-(OH)₂D₃ action. Interestingly, we found that a combined treatment of 1,25-(OH)₂D₃ and GC allowed for a 5-fold reduction of the GC dose while maintaining their anti-inflammatory effects. **Conclusions:** This study has revealed novel immunomodulatory properties of 1,25-(OH)₂D₃ directed against monocyte subsets capable of TNF- α production. In addition, our data suggest that the introduction of 1,25-(OH)₂D₃ to anti-inflammatory therapy would possibly allow for the use of lower doses of GC. © 2015 S. Karger AG, Basel

Introduction

Monocytes and macrophages perform multiple roles in regulating the immune responses underlying allergic and asthmatic inflammation [1]. Monocytes can be divided into three phenotypically and functionally distinct subsets defined by the differential expression of CD14 and CD16

molecules. The majority are CD14⁺⁺CD16⁻ monocytes (referred to as classical), and the other two subsets are CD14⁺⁺CD16⁺ (intermediate) and CD14⁺CD16⁺⁺ (nonclassical) monocytes [2]. Monocyte subsets with CD16 expression are largely expanded in the course of different inflammatory and infectious conditions including asthma, HIV, cardiovascular disease, rheumatoid arthritis and sepsis [3]. We recently demonstrated that nonclassical monocytes, in contrast to other monocyte subsets, are capable of secreting substantial amounts of TNF- α [4]. Classical CD14⁺⁺CD16⁻ monocytes represent the least mature subset; some of these cells could become intermediate and nonclassical monocytes represent the most mature developmental stage [2]. In concert with this notion, these CD14⁺CD16⁺⁺ monocytes share many phenotypic and functional features of macrophages.

We previously demonstrated that numbers of nonclassical CD14⁺CD16⁺⁺ and, to a lesser extent, CD14⁺⁺CD16⁺ monocytes in asthmatic patients can be efficiently decreased by short-term glucocorticoid (GC) treatment [5]. Recently, we confirmed this observation in SIV-infected nonhuman primates [4]. GC-induced depletion of CD16⁺ monocytes occurs in a caspase-dependent manner and is related to increased levels of GC receptors in CD16⁺ monocytes [6]. A recent study by Zhang et al. [7] revealed that some of the anti-inflammatory actions of GC directed at monocytes can be enhanced by treatment with the active form of vitamin D, i.e. 1 α ,25-dihydroxyvitamin D₃ [1,25-(OH)₂D₃]. Here, we demonstrated that 1,25-(OH)₂D₃ itself was capable of depleting of CD16⁺ monocytes and decreasing TNF- α production by monocytes. Moreover, joint treatment with 1,25-(OH)₂D₃ and GC allowed for a substantial reduction of the GC dose while maintaining anti-inflammatory effects.

Methods

Study Subjects

All recruited individuals gave their informed consent prior to their inclusion in the study. The study group consisted of nonsmoking, healthy Caucasian individuals and asthmatic patients. There was no statistical difference in age (31.6, SD $\pm$ 11.5 years vs. 39.6, SD $\pm$ 10.8 years, p = 0.12) and gender distribution (male/female: 51%/49% vs. 56%/44%, p = 0.80) between healthy and asthmatic subjects, respectively. At the initial diagnosis, asthmatic patients were classified with persistent mild or moderate asthma. At the time of the study, their asthma was classified as well-controlled (according to the guidelines of the Global Initiative for Asthma, revised in 2006) following treatment over 3–6 months with inhaled GC (400 μ g of budesonide or 250 μ g of fluticasone per day) and inhaled long-acting beta agonists (24 μ g of formoterol or 100 μ g of salmeterol per day). Blood was collected between the months of October and March.

Cell Culture

EDTA-anticoagulated whole-blood samples obtained from healthy volunteers and asthmatic patients were used to isolate peripheral blood mononuclear cells (PBMC) by density gradient sedimentation. The PBMC (1×10^6) were incubated for 24 h at 37°C in 5% CO₂ in the presence of 1 μ M or 0.2 μ M of methylprednisolone (MP; methylprednisolone sodium succinate, Solu-Medrol, Pfizer Europe, UK) and/or 100 nM 1,25-(OH)₂D₃ (calcitriol, Cayman Chemical, Ann Arbor, Mich., USA). Similar incubation was conducted in the presence of 10 μ M of the caspase inhibitor, zVAD-fmk (Z-VAD-FMK, Kamiya Biomedical Company, Seattle, Wash., USA) or 3 μ M of mifepristone (Sigma-Aldrich, St. Louis, Mo., USA). For analysis of intracellular TNF- α release, PBMC were cultured in presence of either 1 μ g/ml of lipopolysaccharide (LPS; from *E. coli* 0111:B4, Sigma-Aldrich) or 10 μ g/ml of polyinosinic:polycytidylic acid (TLR3 agonist; Poly (I:C) HMW, InvivoGen, San Diego, Calif., USA). PBMC cultured in medium alone (RPMI 1640, Sigma-Aldrich) supplemented with 10% fetal bovine serum (Gibco, UK) and 1% penicillin/streptomycin (Sigma-Aldrich) served as a negative control. Following incubation for 24 h at 37°C in a humidified 5% CO₂ atmosphere, the cells were gently harvested and washed immediately in phosphate-buffered saline (Biomed Lublin, Poland) to prepare them for extracellular and intracellular staining (described below).

Immunostaining and Flow Cytometric Analysis

PBMC collected after cell culture were stained with 5 μ l of the following monoclonal antibodies: CD14 PE (M5E2), CD14 PerCP (M ϕ P9), CD16 FITC (3G8), HLA-DR APC (TU36) (BD Biosciences, Belgium) and incubated for 30 min in the dark at room temperature. For additional intracellular staining, cells were permeabilized and incubated with monoclonal antibodies directed at TNF- α PE (6401.1111) (BD Biosciences). Flow cytometric data were acquired on a FACSCalibur flow cytometer (BD Biosciences, California, USA) and analyzed with the use of CellQuest software (BD Biosciences, USA) and FlowJo software (Tree Star Inc., Ashland, Ore., USA). Monocyte subsets (CD14⁺⁺CD16⁻, CD14⁺⁺CD16⁺ and CD14⁺CD16⁺⁺) were delineated based on fluorescence-minus-one (FMO) controls and varying levels of expression of CD14 and CD16, and were presented as portions of an entire monocyte population [5].

Statistical Analysis

Statistical analysis was conducted with the use of GraphPad Prism 5 (GraphPad Software Inc., California, USA) and, based on the data distribution, parametric or nonparametric paired tests for repeated measures were used (the paired Student t test or the Wilcoxon matched-pairs test, respectively).

Results

First, we found that *in vitro* treatment of PBMC from healthy and asthmatic patients (at a stable stage of the disease) with 100 nM 1,25-(OH)₂D₃ led to a significant decrease of both CD14⁺⁺CD16⁺ and CD14⁺CD16⁺⁺ monocytes, except for the intermediate CD14⁺⁺CD16⁺ monocytes in the asthmatic patients where the p value reached the level of 0.0625 (fig. 1; online suppl. fig. 1; for all suppl.

Fig. 1. Effects of in vitro treatment with MP and 1,25-(OH)₂D₃ on CD14++CD16+ and CD14+CD16++ monocyte subsets in healthy individuals and asthmatic patients. **a** Gating strategy for delineation of different monocyte subsets (CD14++CD16-, CD14++CD16+ and CD14+CD16++) was based on typical for monocyte FSC/SSC features, positive HLA-DR expression and differential levels of CD14 and CD16 expression. **b** Changes in the percentage of CD16-positive monocyte subsets in PBMC collected from healthy individuals following 24-hour in vitro culture with 1 μM of

MP and/or 100 nM of 1,25-(OH)₂D₃. **c** Changes in the percentage of CD16-positive monocyte subsets in PBMC collected from asthmatic patients following 24-hour in vitro culture with 1 μM of MP and/or 100 nM of 1,25-(OH)₂D₃. **b, c** Representative flow cytometric data (upper rows). The numerical values refer to the frequency of CD14++CD16+ monocytes (out of all monocytes) and CD14+CD16++ monocytes (lower rows). UNST = Unstimulated culture; Vit.D₃ = vitamin D₃. (For figure 1c see next page.)

material, see www.karger.com/doi/10.1159/000380882). Moreover, the addition of 100 nM 1,25-(OH)₂D₃ to the 1 μM GC culture resulted in further enhancement of the monocyte-directed effects observed with the use of GC or 1,25-(OH)₂D₃ alone.

Next, we set out to identify the mechanism of 1,25-(OH)₂D₃-mediated depletion of the CD16-positive subsets. To this end, we cultured PBMC for 24 hours in the presence of 1,25-(OH)₂D₃ and caspase-3, caspase-6 and caspase-7 inhibitors, namely zVAD-fmk. We found

that the inhibition of the caspase pathways resulted in abrogation of the depleting effects of 1,25-(OH)₂D₃ directed at the nonclassical CD14+CD16++ monocytes (fig. 2a). Thus, we demonstrated that a decrease in the frequency of CD16-positive monocytes is associated with the enhancement of proapoptotic pathways mediated by 1,25-(OH)₂D₃. Interestingly, the depletion of nonclassical CD14+CD16++ monocytes (but not intermediate CD14++CD16+ monocytes) by 1,25-(OH)₂D₃ was inhibited in the presence of mifepristone, a GC and progesterone receptor antagonist.

Having found GC and 1,25-(OH)₂D₃ to have similar action, we next wished to explore whether stimulation with lower doses of GC in the presence of 1,25-(OH)₂D₃ could achieve effects comparable to those seen with the use of higher doses of GC. Indeed, we demonstrated that a combined treatment of 5-fold-lower doses of GC and 1,25-(OH)₂D₃ resulted in effects being exerted on both the CD14++CD16+ and CD14+CD16++ monocytes similar to treatment with higher doses of GC (fig. 2b; online suppl. fig. 2).

Finally, we demonstrated that addition of 1,25-(OH)₂D₃ significantly decreased the capacity of monocytes to secrete TNF-α following 24-hour stimulation with LPS (fig. 2d). More detailed analysis of the diminished TNF-α secretion by the different monocyte subsets following stimulation with 100 nM 1,25-(OH)₂D₃ revealed that this effect was most pronounced in the intermediate CD14++CD16+ monocytes. Similarly, we found that addition of 100 nM 1,25-(OH)₂D₃ significantly abrogated monocyte-related TNF-α secretion induced by poly(I:C) that is similar to double-stranded RNA and is known to interact with Toll-like receptor 3 (TLR3). In some contrast to LPS stimulation, however, abrogating effects of 1,25-(OH)₂D₃ on poly(I:C) stimulation were mostly seen in classical and intermediate but not non-classical monocytes. Nevertheless, despite differential pattern of responses of non-classical monocytes to co-stimulation with 1,25-(OH)₂D₃ and LPS or poly(I:C), the overall abrogating effect of active form of vitamin D₃ on total release of TNF-α by all monocytes remained similar for both TLR3 and TLR4 ligands.

Fig. 2. **a** Effects of inhibition of caspase-dependent apoptotic pathway with zVAD-fmk (zVAD) and antagonization of GC and progesterone receptor (mifepristone, MF) on 1,25-(OH)₂D₃-dependent actions against CD14⁺⁺CD16⁺ and CD14⁺CD16⁺⁺ monocytes in healthy subjects and asthmatic patients. **b** In vitro treatment of PBMC with a low dose of GC and 100 nM of 1,25-(OH)₂D₃. Effects of in vitro 24-hour culture of PBMC with a 5-fold-lower dose of MP (0.2 μM)

supplemented or not with 100 nM of 1,25-(OH)₂D₃ on the frequency of CD14⁺⁺CD16⁺ and CD14⁺CD16⁺⁺ monocytes in healthy subjects and asthmatic patients. **c** Gating strategy for delineation of TNF-α-positive monocytes. **d** Effects of 24-hour co-culture with 1,25-(OH)₂D₃ on LPS/poly(I:C)-induced secretion of TNF-α by all monocytes and the different subsets. UNST = Unstimulated culture; Vit.D₃ = vitamin D₃.

(For figure 2d see next page.)

2

Discussion

Here we identified novel independent and steroid-enhancing properties of 1,25-(OH)₂D₃. To date, there is a growing body of evidence indicating strong relationships between vitamin D deficiency and numerous inflammatory disorders, including asthma [8–10]. Based on the current epidemiologic data and known anti-inflammatory properties of vitamin D, its supplementation is frequently advised for patients with various clinical conditions. The most important tool used for evaluation of the so-called ‘vitamin D status’ is an assessment of concentrations of 25-hydroxycholecalciferol (calcidiol). This is due to the longer half-time of calcidiol compared to its biologically active metabolite calcitriol which was used in this study. Current recommendations indicate that the optimal serum calcidiol concentration should be >75 ng/ml [11]. Unfortunately, these values are very rarely reached in Western societies [12]. Therefore, many experts advise that individuals with low vitamin D concentrations should take up to 2,000–4,000 IU of calcidiol per day [13]. In fact, this is exactly the dose that, following conversion to calcitriol, would give the concentration of 100 nM (the same as was used in our experiment) [14].

We demonstrated unknown mechanisms of the anti-inflammatory actions of 1,25-(OH)₂D₃ with regard to its effects on monocyte subsets which were previously demonstrated as significantly enriched in asthmatic patients

[5]. Interestingly, however, despite an identical pattern of monocyte-related responses to vitamin D₃ in healthy and asthmatic individuals, we observed that some of the effects of 1,25-(OH)₂D₃ on the monocyte subsets were slightly less pronounced in the asthmatic subjects. This was probably not caused by differences in endogenous vitamin D levels as healthy individuals and asthmatic patients were found to have similarly low serum calcidiol concentrations (data not shown). In fact, one of the limitations of this study is the lack of more detailed analysis of the reasons for the existence of these small but intriguing differences between the healthy and the asthmatic subjects. Therefore, further studies are warranted to explore whether immune cells from asthmatic subjects could be more resistant to vitamin D-related actions.

We provided evidence that 1,25-(OH)₂D₃ alone and in combination with GC exerts proapoptotic effects on these monocyte subsets (that bear the highest capacity to produce proinflammatory TNF-α) and, in turn, decreases the total TNF-α production by the monocytes. Recently, Yilmaz et al. demonstrated that experimental antagonization of TNF-α actions resulted in preservation of epithelium and basement membrane layer in asthmatic subjects [15]. Further studies are warranted to clarify whether our findings of decrease of TNF-α-secreting monocytes can, at least in part, account for positive effects of vitamin D in asthma. Interestingly, our experiments with pan-caspase inhibitors (inhibiting caspase-3, cas-

pase-6 and caspase-7) and GC antagonist indicate that, despite similarities, the effects of 1,25-(OH)₂D₃ on intermediate and nonclassical monocyte subsets can differ. Based on the results of our study, we can hypothesize that, in contrast to GC, the proapoptotic effects of 1,25-(OH)₂D₃ directed at intermediate CD14⁺⁺CD16⁺ monocytes might be exerted by the use of pathways other than those related to caspase-3, caspase-6 and caspase-7. However, we did not test this hypothesis in sufficient detail and it should be explored in future studies.

Our data complement the findings of Zhang et al. [7] and our previous studies on the GC-mediated depletion of CD16-positive monocytes in asthmatic patients [5]. Here, we demonstrated that 1,25-(OH)₂D₃ exerts effects that are similar to those observed with GC. Importantly, we found that combination of GC and 1,25-(OH)₂D₃ facilitated the use of lower doses of GC while maintaining comparable anti-inflammatory effects. In fact, there is a large body of evidence indicating that in vivo vitamin D treatment improves clinical status, lung function and steroid responsiveness in asthmatic patients [16, 17]. Our results do not justify a conclusion that the beneficial effects of vitamin D observed in the asthmatic patients could have been related to its effects on proinflammatory monocytes. On the other hand, they do provide a perti-

nent indication for the further testing of the effects of interactions between vitamin D and monocytes on clinical outcome.

Conclusions

Our data suggest that the introduction of vitamin D to antiasthmatic therapy would possibly allow for the use of lower doses of GC in asthma patients. Further clinical studies are warranted to address this possibility in more detail. However, given the widely known side effects of GC administration, any interventions aimed at dose reduction are of clinical benefit and interest.

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Disclosure Statement

The authors declare that they have no conflicts of interest.

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