HUMAN NEUTROPHILS EXPRESS MESSENGER RNA OF VITAMIN D RECEPTOR AND RESPOND TO 1α,25-DIHYDROXYVITAMIN D₃

Katsushi Takahashi,¹,²* Yasunori Nakayama,¹ Hideki Horiuchi,¹ Tomohiro Ohta,¹ Keiji Komoriya,¹ Hitoshi Ohmori,² and Takashi Kamimura¹

¹Pharmaceuticals Development Laboratories, Teijin Institute for Bio-Medical Research, 4-3-2 Asahigaoka, Hino, Tokyo 191-8512, Japan
²Department of Biotechnology, Okayama University, Okayama, Japan

ABSTRACT

1α,25-Dihydroxyvitamin D₃ (1,25(OH)₂D₃) has been shown to modulate the production of various cytokines or the expression of certain differentiation markers in human T cells or monocytes. Its effects on neutrophils, however, are poorly understood. In this paper, we show several lines of evidence indicating that neutrophils express functional vitamin D receptors (VDR). Sort-purified neutrophils from human peripheral blood expressed VDR mRNA at a level comparable to that of monocytes. As reported to occur in monocytes, protein expression of CD14 on the cell surface of neutrophils was augmented when the cells were incubated with 1,25(OH)₂D₃. To investigate the physiological roles for VDR in neutrophils, we investigated possible modulating effects of 1,25(OH)₂D₃ on the expression of several genes in lipopolysaccharide-stimulated neutrophils by using differential display analysis. Of the genes we identified, trappin-2/elafin/SKALP, which was originally reported to

*Corresponding author. Fax: +81-42-587-5517; E-mail: kat.takahashi@teijin.co.jp

DOI: 10.1081/IPH-120014721 0892-3973 (Print); 1532-2513 (Online)
be an inhibitor of elastase, was induced in neutrophils by lipopolysaccharide, but was suppressed significantly in the presence of 1,25(OH)$_2$D$_3$. Under the same conditions, interleukin-1β expression was also inhibited. These findings suggest that 1,25(OH)$_2$D$_3$ has a potential to affect the inflammatory process by modulating the expression of neutrophil genes.

INTRODUCTION

1α,25-Dihydroxyvitamin D$_3$ (1,25(OH)$_2$D$_3$), a biologically active metabolite of vitamin D$_3$, has various pleiotropic effects, including the control of calcium homeostasis$^[1]$ and promotion of the differentiation of hematopoietic cells$^[2]$ and keratinocytes$^[3]$ by inducing the expression of various genes. It also directly inhibits the expression of certain other genes, such as the interleukin (IL)-2 and granulocyte macrophage-colony stimulating factor (GM-CSF). All of these effects are mediated by its specific nuclear receptor, the vitamin D receptor (VDR), which, upon binding the ligand, is recruited to specific nucleotide sequences in the promoter regions of the target genes.$^[4-6]$ Neutrophils contribute to the undesirable sequelae of sepsis, including the multiple organ dysfunction syndrome and the acute respiratory distress syndrome.$^[7]$ 1,25(OH)$_2$D$_3$ has been shown to exert protective effects against sepsis in mice.$^[8]$ These effects have been attributed to the inhibitory effects of the vitamin on IL-8 production in monocytes/macrophages.$^[9]$ On the other hand, the effects of 1,25(OH)$_2$D$_3$ on neutrophil function are poorly understood. Thus, we investigated the physiological effect of 1,25(OH)$_2$D$_3$ on neutrophils derived from human peripheral blood. In the present report, we show that neutrophils expressed a level of VDR mRNA comparable to that in monocytes and that the expression of several neutrophil genes was modulated in response to 1,25(OH)$_2$D$_3$.

MATERIALS AND METHODS

Cell Culture

Human peripheral blood was collected from healthy volunteers in heparinized tubes. Peripheral blood mononuclear cells (PBMC) and polymorphonuclear cells (PMN) were separated by discontinuous density-gradient centrifugation using Ficoll-Paque$^\text{®}$ (Pharmacia, Upssala, Sweden) and by dextran sedimentation. Typical cell populations of the PBMC preparations were ~80% lymphocytes (CD15$^-$, CD33$^-$, CD45$^+$), ~15% monocytes (CD15$^{\text{dim}}$, CD33$^{\text{bright}}$, CD45$^+$), and ~5% granulocytes (CD15$^{\text{bright}}$, CD33$^{\text{dim}}$, CD45$^+$) as determined by flow cytometry. The PMN preparations were <0.5% lymphocytes, <0.1% monocytes, and >99%
granulocytes. The PBMC and PMN were suspended at 10^6 cells/mL in medium (RPMI-1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 U/mL penicillin, and 50 mg/mL streptomycin). Separated cells and whole blood (2 × 10^6 cells, 0.2 mL) were cultured in 96-well flat-bottomed plastic plates (Becton Dickinson, Franklin Lakes, NJ) for 4 h in a humidified incubator having an atmosphere of 5% CO₂ at 37°C. 1,25(OH)₂D₃ (Teijin Ltd., Tokyo, Japan) was dissolved in ethanol and added at the final concentrations of 10⁻⁷ M and 0.1% ethanol at the beginning of the culture period. LPS (E. coli 026:B6, DIFCO Laboratories, Detroit, MI) was used at the final concentration of 1 µg/mL. Recombinant human TNFα, IL-1β, IL-8, G-CSF, and GM-CSF (Genzyme Diagnostics, Cambridge, MA) were used at the final concentration of 50 ng/mL.

Cell Sorting

PMN and PBMC (10⁷ cells/mL in the medium) were stained with anti-human CD15-FITC (clone: HI98, Pharmingen, San Diego, CA, USA), anti-human CD33-PE (clone: WM53, Pharmingen), or anti-human CD45-Cy-Chrome® (clone: HI30, Pharmingen) for 30 min at 4°C, washed twice with cold PBS containing 1% fetal bovine serum, and subjected to flow cytometric analysis and the sorting procedure. Fluorescence-activated cell sorting was performed on a FACSCalibur® (Becton Dickinson). Neutrophils (CD15⁺, CD33dim, CD45⁺) and monocytes (CD15⁻, CD33bright, CD45⁻) were sorted and immediately processed for RNA isolation.

Flow Cytometric Analysis of CD14 Expression

Human whole blood was treated with 10⁻⁷ M 1,25(OH)₂D₃ for 24 h. Cells in whole blood were stained by treatment with anti-human CD14-FITC (clone: M5E2, mouse IgG2a, Pharmingen) or mouse IgG2a-FITC (clone DAK-G05, Dako, Denmark), and anti-human CD33-PE (Becton Dickinson) for 30 min at 4°C, and then the erythrocytes were lysed. The resulting cells were fixed with 1% formaldehyde in PBS and then analyzed by the FACSCalibur® (Becton Dickinson). Neutrophils and monocytes were identified on the basis of CD33 expression and the scattergram of front scatter versus side scatter.

Reverse Transcriptase-Polymerase Chain Reaction Analysis

Polyadenylated RNA was directly prepared from 4 × 10⁴ sorted neutrophils or monocytes by using an oligo-dT cellulose column (Quick-Prep® Micro mRNA Purification Kit, Amersham Pharmacia Biotech).
First-strand cDNA was prepared from the mRNA (corresponding to $3 \times 10^4$ cells) and a random hexamer ($0.2 \mu g$) with Ready-To-Go® You-Primed First Strand Beads (Amersham Pharmacia Biotech). The prepared cDNA was amplified in a 25-µL reaction mixture containing primers ($0.2 \mu M$) by using a HotStarTaq PCR Master Mix Kit (Qiagen, Hilden, Germany) and a DNA thermal cycler (model 9600; Perkin-Elmer Corporation, Norwalk, CT). Prior to the PCR cycles, the enzyme was activated at 95°C for 15 min. One PCR cycle consisted of denaturation at 94°C for 20 s, annealing at 55°C (VDR, CD14, IL-8 receptor A: CDw128a, and β-actin) for 30 s, and extension at 72°C for 30 s. PCR was conducted for 35 to 40 cycles. The final extension was allowed to continue at 72°C for 5 min. After completion of the PCR, 5 µL of each reaction mixture was analyzed on a 2% NuSieve agarose gel (FMC Bioproducts, Rockland, ME) and visualized by ethidium bromide staining. The primers used for the PCR were as follow: for VDR, sense 5'-GACCTACGACCCACCTACT-3' and antisense 5'-TAGGGTCACAGAAGGGTCAT-3'; for CD14, sense 5'-TGCCGCTGTGTAGAAAGAAG-3' and antisense 5'-CTGAGGTTCGGAGAAGTTGC-3'; for HLA-DR, sense 5'-GGGCTATCAAAGAA-GAACAT-3' and antisense 5'-AGAAACACCACACCTTCCAT-3'; for CDw128a, sense 5'-AGGATGTGGGGAAGTTAGGA-3' and antisense 5'-ATGTTGCTCGGATGTGGTC-3'; for β-actin, sense 5'-CAGGACCGCTGCT-3' and antisense 5'-TCCTTCTGCACTCCTGT-CGGCA-3'. Primers were chosen to exclude or to distinguish by size genomic DNA amplicons, except for CDw128a. β-Actin competitor (Takara, Kyoto, Japan) was used as the internal standard.

For real-time PCR analysis, total RNA was prepared from 0.2 mL of whole blood or from $2 \times 10^5$ PMN and PBMC by using RNeasy® Mini Kits and an RNase-Free DNase Set (Qiagen). First-strand cDNA was prepared from the total RNA (50 ng) and oligo-dT15 primers (20 pmol) with Sensiscript® RT Kit (Qiagen) by using the standard protocol. cDNA was diluted to 10 volumes with 1 x TE buffer. cDNA (2 µL) was amplified in the presence of 5 µL of TaqMan universal master mix (Applied Biosystems, Foster City, CA), 2 pmol of gene-specific TaqMan probe, and forward and reverse primers in a final volume of 10 µL. The primers and TaqMan probes used for the real-time PCR were as follow: for trappin-2/elafin/SKALP, sense 5'-TGCTGTGAAGCTCTTGCG-3', anti-sense 5'-CACAGGTGCA-GGAAGGACC-3', and probe 5'-FAM-TGGCCTGTGTTTCCGACT-CGGT-GAAG-TAMRA-3'; for IL-1β, sense 5'-GCTGCTCTGGGATATTCA-3', antisense 5'-TCCTGCACTCCTTGTGTC-3', and probe 5'-FAM-CATTGCTCAAGTGTCTGAAGCAGCCATG-TAMRA-3'; for TNFα, sense 5'-ACATACTGACCCACGTTCA-3', antisense 5'-GTCCCGGATCTGCTTTC-3', and probe 5'-FAM-TCCTCCACAGACA-CATTG-3'. Samples underwent the following stages: stage 1, 50°C for 2 min; stage 2, 95°C for 10 min; and stage
3, 95°C for 15 s followed by 60°C for 1 min. Stage 3 was repeated 40 times. Gene-specific PCR products were measured by means of a GeneAmp® 5700 Sequence Detection System (Applied Biosystems) continuously during 40 cycles. Target gene expression was normalized between different samples based on the values of the expression of the β-actin.

**Differential Display Analysis**

(a) Primers. Arbitrary primers used were 32 random 20-mer oligodeoxynucleotides. Anchored primers were designed by adding the CCCGGATCC sequence (containing a Bam site) to 3 kinds of one-base anchored 15-mer oligo dT. Ninety-six different primer pairs were used for the differential display analysis. (b) PCR amplification and band separation. The cDNA, prepared from LPS-stimulated PMN with or without 1,25(OH)²D₃ as described above, was amplified in a 10-µL reaction mixture containing arbitrary and anchor primers (1 µM) by using a HotStarTaq PCR Master Mix Kit (Qiagen, Hilden, Germany) and a DNA thermal cycler (model 9600; Perkin-Elmer Corporation, Norwalk, CT). The amplified cDNAs were then separated on a DNA sequencing gel (6% polyacrylamide, non-denaturing). At the end of the electrophoresis, the sequencing gel was stained with 0.01% SYBR® Green I in 1×TBE for 30 min at room temperature, and then examined with a FLA-3000G fluoro image analyzer (Fuji Film, Tokyo). (c) Recovery and reamplification of target cDNA fragments. The target bands, whose intensities differed by more than 2-fold between control and 1,25(OH)²D₃-treated samples, were cut out from the gels and directly reamplified in a 50-µL reaction mixture containing the corresponding primers (0.2 µM). The cycling parameters were as follow: 95°C for 15 min for the enzyme activation, 95°C for 15 s, 55°C for 1 min, 72°C for 1 min for 20 cycles, and finally post-extension at 72°C for 5 min. The PCR products were purified by 2% agarose gel electrophoresis, and sequenced by using an ABI PRISM BigDye terminator cycle sequencing ready reaction kit and 310 Genetic analyzer (Applied Biosystems). Each sequence was searched against non-redundant databases of the National Center for Biotechnology Information (NCBI) by the Basic Local Alignment Search Tool (BLAST) program. Matches to known genes were confirmed by quantitative real-time PCR analysis.

**Statistical Analyses**

All statistical analyses were performed by using SAS® System Release 6.12 (SAS Institute Inc., Cary, NC) with EXSAS version 5.10 (Arm, Osaka,
Japan). The statistical significance of the mRNA expression difference between groups was determined by the nonparametric Wilcoxon test.

RESULTS

Expression of VDR mRNA in Neutrophils

Neutrophils and monocytes were prepared from the blood sample of the same donor by cell sorting. RT-PCR analysis showed that RNA extracted from the purified neutrophils (CD15^+^, CD33^{dim}) contained a very low level of the transcripts for CD14 and a high level of those for the IL-8 receptor, i.e., CDw128a, but no detectable HLA-DRα mRNA (Fig. 1). On the other hand, purified monocytes (CD15^-^, CD33^{bright}) expressed an appreciable level of the transcripts for CD14 and HLA-DRα, but no detectable mRNA for CDw128a. These results show no detectable cross contamination between the two cells preparations. An interesting observation was that the neutrophils expressed VDR at a level comparable to that expressed by monocytes (Fig. 1). As the role for VDR in neutrophils remained to be elucidated, additional experiments were performed to investigate whether the expressed VDR was functional and what types of biological effects might be mediated by the receptor.

Figure 1. Expression of VDR mRNA in human neutrophils. Expression of human VDR, CD14, HLA-DRα, CDw128, and β-actin in sort-purified human neutrophils and monocytes were analyzed by RT-PCR as detailed in Materials and Methods.
Effect of 1,25(OH)₂D₃ on CD14 Expression on Neutrophils

As VDR mRNA was found to be expressed in neutrophils, we first investigated whether 1,25(OH)₂D₃ could deliver some signal via VDR. In monocytes, it has been reported that the expression of CD14 is augmented by treatment with 1,25(OH)₂D₃. Thus, we tested whether CD14 expression would also be enhanced by 1,25(OH)₂D₃ in neutrophils. Flow cytometric analysis revealed that the level of CD14 in neutrophils, which was less than 10% of that in monocytes, was significantly augmented in the presence of 1,25(OH)₂D₃ (Table 1). Thus, these data suggest that neutrophils indeed expressed functional VDR.

Effect of 1,25(OH)₂D₃ on Trappin-2/elafin/SKALP mRNA Expression in Neutrophils

To investigate the effects of 1,25(OH)₂D₃ on the expression of various genes, we performed an extensive comparison of the cDNA preparations from neutrophils that had been stimulated with LPS for 4 h in the presence or absence of 10⁻⁷ M 1,25(OH)₂D₃. We analyzed the sequences of 38 PCR-amplified bands whose intensities differed by more than 2-fold between the control and the treated samples. By means of the BLAST computer program the sequences were compared with the human non-redundant DNA sequences previously reported in the GenBank, EMBL, and DDBJ databases. As a result, we found that the expression of 6 known genes, which had not been reported to have any relation to 1,25(OH)₂D₃, was modulated by 1,25(OH)₂D₃ in neutrophils. One of these genes, trappin-2/elafin/SKALP, which was originally reported to be an inhibitor of elastase, was suppressed markedly in the presence of 1,25(OH)₂D₃ (Fig. 2). Among peripheral blood leukocytes, this gene was specifically induced in neutrophils that had been stimulated by LPS, but not in mononuclear cells (Fig. 3). Of various stimulants examined, LPS, TNFα, and IL-1β were effective in inducing trappin-2 expression (Fig. 4). Next, we assessed the inhibitory effect of 1,25(OH)₂D₃ on the expression of trappin-2, TNFα, and IL-1β induced by these stimulants. In the case of LPS-stimulation, 1,25(OH)₂D₃ significantly inhibited mRNA expression of TNFα and IL-1β, as well as that of trappin-2.

Table 1. Expression of CD14 on Human Peripheral Blood Leukocytes

<table>
<thead>
<tr>
<th>1,25(OH)₂D₃ (10⁻⁷ M)</th>
<th>Neutrophil</th>
<th>Monocyte</th>
</tr>
</thead>
<tbody>
<tr>
<td>−</td>
<td>1.18 ± 0.06</td>
<td>2.84 ± 0.08</td>
</tr>
<tr>
<td>+</td>
<td>1.62 ± 0.04</td>
<td>3.19 ± 0.04</td>
</tr>
</tbody>
</table>

Data are represented as the logarithm of the geometric mean fluorescence intensity ± S.D. of triplicate cultures. Data from 1 individual, representative of the data from a total of 4 individual donors, is shown.
(Table 2). On the other hand, stimulation with TNFα or IL-1β led to the inhibition of the expression of both IL-1β and trappin-2, but not to that of TNFα. Taken together, these data indicate that IL-1β was inhibited by 1,25(OH)₂D₃ in a similar fashion as trappin-2.
DISCUSSION

Neutrophils have a key role in inflammatory processes, not only by migrating to inflammation sites, but also by producing proinflammatory cytokines. We previously reported that 1,25(OH)₂D₃ had a protective effect on an animal model of sepsis, in which neutrophils are known to be involved in organ dysfunction. The effects of 1,25(OH)₂D₃ on leukocytes had been analyzed in lymphocytes or monocytes/macrophages. On the other hand, the effects on neutrophils are poorly understood. In the present paper, we revealed the modulating effects of 1,25(OH)₂D₃ on some gene expressions in human peripheral blood neutrophils, suggesting that neutrophils have a potential to respond to 1,25(OH)₂D₃.

McTernan et al. showed that the differentiation of HL-60 cells into neutrophils resulted in the reduction in the level of VDR expression. However, it has not been reported whether VDR expression is downregulated with differentiation of normal neutrophils. Our data show that peripheral blood neutrophils expressed a level of VDR mRNA comparable to that expressed by monocytes. Thus, no drastic alteration in VDR level may occur during normal myeloid differentiation.

CD14 functions as a receptor for the complex between LPS and LPS-binding protein, thereby playing a key role in LPS-induced inflammatory responses to Gram-negative bacterial infections. As CD14 on monocytes had

![Figure 3](image-url)
already been shown to be enhanced by 1,25(OH)₂D₃,[14] we examined whether the CD14 expression on neutrophils would also be modulated by 1,25(OH)₂D₃. As a result, CD14 expression on neutrophils was augmented in the presence of 1,25(OH)₂D₃. This augmentation may have been due to the direct action of the vitamin on neutrophils because the effect was reproducible using purified neutrophil preparations. Thus, it is suggested

Figure 4. Expression of trappin-2 mRNA in human PMN in response to various stimulants. Trappin-2 expression in human PMN incubated with various stimulants for 4 h was assessed by quantitative TaqMan PCR. Data are expressed as relative amount of trappin-2 mRNA to β-actin for each individual. The compiled data represent the mean ± SD for 4 individual donors.

Table 2. Inhibitory Effects of 1,25(OH)₂D₃ on mRNA Expression in Human Neutrophils

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>Trappin-2 % of control</th>
<th>p value*</th>
<th>IL-1β % of control</th>
<th>p value</th>
<th>TNFα % of control</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS</td>
<td>75.9 ± 9.8</td>
<td>0.008</td>
<td>85.6 ± 16.8</td>
<td>0.023</td>
<td>78.7 ± 10.3</td>
<td>0.008</td>
</tr>
<tr>
<td>TNFα</td>
<td>82.5 ± 14.5</td>
<td>0.125</td>
<td>85.3 ± 8.5</td>
<td>0.063</td>
<td>103.5 ± 13.3</td>
<td>1.000</td>
</tr>
<tr>
<td>IL-1β</td>
<td>80.0 ± 6.9</td>
<td>0.031</td>
<td>86.5 ± 25.0</td>
<td>0.156</td>
<td>102.5 ± 34.1</td>
<td>0.688</td>
</tr>
</tbody>
</table>

Trappin-2, IL-1β, and TNFα expression in human neutrophils incubated with various stimulants for 4 h were assessed by quantitative TaqMan PCR. Target gene expression was normalized between different samples based on the values of the expression of the β-actin. The expression level in the presence of 1,25(OH)₂D₃ is presented as a percentage of control (in the absence of 1,25(OH)₂D₃) for each individual. The compiled data represent the mean ± SD for 4 individual donors. *Statistical significance were assessed by the nonparametric Wilcoxon test.
that 1,25(OH)$_2$D$_3$, unlike glucocorticoid, augments some immune responses to bacterial infection.

Furthermore, to investigate the effects of 1,25(OH)$_2$D$_3$ on neutrophils, which cells are especially involved in inflammatory responses, we performed differential display analysis, and identified several genes modulated by 1,25(OH)$_2$D$_3$ in LPS-stimulated neutrophils.

Trappin-2/elafin/SKALP had been originally reported as an inhibitor of neutrophil elastase.$^{[15]}$ Because trappin-2 is highly expressed in psoriasis lesions,$^{[16]}$ it is thought to be useful as a diagnostic marker of such disorders that are characterized by dermal neutrophil infiltration.$^{[17]}$ Major cells that are responsible for trappin-2 expression in the disease are dermal keratinocytes, which have been demonstrated to produce trappin-2 in response to TNF-α or IL-1β in vitro.$^{[18]}$ We report here for the first time that trappin-2 was also expressed in neutrophils that had been stimulated by LPS, TNF-α, or IL-1β, but not in mononuclear cells. Although the physiological significance of these findings remains to be clarified, a fraction of trappin-2 expression detected in psoriasis may possibly come from neutrophils. This trappin-2 expression in neutrophils was significantly suppressed by 1,25(OH)$_2$D$_3$. In addition, IL-1β expression was simultaneously inhibited under the same culture conditions. The 5′-upstream region of the trappin-2 gene has AP-1 and NF-κB binding motifs,$^{[19]}$ and 1,25(OH)$_2$D$_3$ has been shown to antagonize the transcriptional activity of AP-1$^{[19]}$ or NF-κB.$^{[20]}$ Therefore it is possible that 1,25(OH)$_2$D$_3$ directly suppressed the gene expression of trappin-2. On the other hand, our results suggest that trappin-2 may have been suppressed indirectly by the inhibition of IL-1β. Brandolini et al. showed that a brief exposure of neutrophils to IL-1β induced a potentiation of elastase release.$^{[21]}$ Taken together, the available information suggests that IL-1, produced by LPS-stimulated neutrophils, can induce elastase release, which may be followed by the expression of trappin-2 via an autocrine mechanism. 1,25(OH)$_2$D$_3$ may have the potential to inhibit these inflammatory responses by inhibiting IL-1β production.

Collectively, our data demonstrate that neutrophils have the ability to respond to 1,25(OH)$_2$D$_3$, and this finding led to additional evidence that 1,25(OH)$_2$D$_3$ can affect inflammatory processes by modulating the expression of genes in neutrophils.

REFERENCES


16. Tanaka, N.; Fujioka, A.; Tajima, S.; Ishibashi, A.; Hirose, S. Elafin is Induced in Epidermis in Skin Disorders with Dermal Neutrophilic Infiltration:


