<Research Article>

Genistein promotes phagocytic phenotypes, except those associated with monocytic marker CD14, in 1*α*,25-dihydroxyvitamin D_3-induced monocytic differentiation of HL60 cells

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Summary Genistein is a soybean isoflavone and a potential natural supplement for cancer therapy because of its anti-proliferative effects and ability to induce apoptosis in leukemic cells in vitro and in vivo. A couple of reports suggest that genistein affects 1α,25-dihydroxyvitamin D_3 (VD_3)-induced monocytic differentiation. However, this effect has not been characterized well, and prior studies used supra-dietarily achievable genistein doses. Therefore, we analyzed the additional phenotypes affected by a dietarily achievable concentration of genistein (10 μmol/L) on VD_3-induced monocytic differentiation using HL60 cells. Genistein increased the G_0/G_1 phase ratio in the cell cycle and inhibited cell growth. Furthermore, it upregulated the protein and mRNA expression of both CD11b and CYBB (a Nox2 subunit) and enhanced the phagocytic ability of VD_3-treated HL60 cells. However, genistein did not affect the expression of CD14, a monocytic marker. These results suggest that genistein can preferentially promote common phenotypes in phagocytes, such as neutrophils and monocytes, but not monocyte-specific ones, in the context of VD_3-induced monocytic differentiation.

Keywords: 1α,25-Dihydroxyvitamin D_3, CD14, Genistein, HL60 cells, Phagocytic phenotype

1. Introduction

Monocytes and neutrophils are important cells of the innate immune system[1]. Monocytes are transported via blood to the target tissue, where they differentiate into macrophages that eliminate recognized foreign antigens via phagocytosis, bactericidal action, and degradation[2]. Monocytes are derived from hematopoietic stem cells (HSCs) in the bone marrow and originate from granulocyte and macrophage progenitors (GMPs)[3]. During differentiation of a monocyte/macrophage lineage, the monocytic marker CD14 and phagocytic markers CD11b and NADPH oxidase 2 (Nox2) are expressed, and phagocytic and bactericidal activities are activated[4,5]. CD14 serves as a co-receptor of Toll-like receptor 4 (TLR4) to amplify the sensitivity of TLR4 to lipopolysaccharides (LPS), thereby facilitating an innate immune response[6]. CD11b functions as a complement receptor component to accelerate phagocytosis of the C3bi-coated antigen such as bacteria[7]. Nox2, which is composed of gp91phox (CYBB), p22phox, p40phox, p47phox, p67phox, and rac2, produces superoxide anions to kill pathogens[8]. Genetic defects in HSCs or

GMPs can result in abnormal proliferation and differentiation (i.e., leukemia)[9]. In contrast to traditional leukemia therapies that kill leukemic cells using anticancer agents and radiation, differentiation therapies force immature leukemia cells to develop into normal mature cells[10,11]. Differentiation therapy using all-trans-retinoic acid (ATRA), which can induce granulocytic differentiation of myeloid leukemia cells, has been shown to improve the prognosis of acute promyelocytic leukemia (APL)[10,11]. Additionally, 1α,25-dihydroxyvitamin D_3 (VD_3) may be suitable for APL therapy because it can induce monocytic differentiation[12]. However, because of the side effects of therapeutical VD_3, e.g., systemic hypercalcemia during treatment, there exists a need to develop or find compounds, including natural ones, that can promote differentiation with minimal side effects[10].

Genistein is a soybean isoflavone and a potential natural supplement for cancer therapy because of its anti-proliferative effects and ability to induce apoptosis in leukemic cells *in vitro* and *in vivo*[13,14]. HL60 cells, established from samples obtained from patients with APL, can be differentiated into neutrophils and monocytes using ATRA and VD_3, respectively[15,16]. Using cell surface marker analysis, genistein was shown to promote ATRA-induced neutrophil differentiation in HL60 cells[17]. Although a couple of reports suggest that genistein affects monocytic differentiation when used in a supra-dietarily achievable dose as a protein kinase inhibitor[18,19], the effect of a physiological dose on VD_3-induced monocytic differentiation is yet to be understood completely.

Therefore, in this study, we performed an in-depth analysis of the effects of dietarily achievable doses of genistein on VD_3-induced monocytic differentiation, with a focus on cell growth, cell cycle, expression of cell surface antigens (CD11b, CYBB, and CD14), and expression of mRNA (CD11b and CYBB).

2. Materials and methods

Chemicals

HL60 cells (human promyelocytic leukemic cell line) were obtained from the RIKEN Cell Bank (catalogue number: RCB0041). Fetal bovine serum, *Staphylococcus aureus* (strain Wood without protein A) BioParticles, and fluorescein conjugate were purchased from Thermo Fisher Scientific (Waltham, MA, USA). RPMI 1640 medium was purchased from Merck KGaA (Darmstadt, Germany). 1α,25-Dihydroxyvitamin D_3 was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Genistein was purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan).

Cell culture and treatment

HL60 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere of 95% air and 5% CO_2. HL60 cells (1.0×10^5 cells) were induced to differentiate into monocytes with 100 nmol/L VD_3. Genistein (10 µmol/L) was added 30 min after initiation of monocytic differentiation using VD_3.

Cell viability and cell growth

Cell number and viability were determined using a Neubauer microchamber (Sunlead Glass Corp, Koshigaya, Japan) with a light microscope, after 1 min of treatment with 0.2% trypan blue dye solution (FUJIFILM Wako Pure Chemical Corporation).

Cell cycle assay

HL60 cells (1.0×10^6 cells) were fixed overnight (>18 h) at 4°C in ice-cold 70% ethanol. The sample was then combined with staining solution (50 µg/mL propidium iodide, 100 Kunitz units/mL RNase A, and 0.1% glucose in phosphate-buffered saline) and incubated for 40 min at 20–25°C. Each sample was analyzed within 24 h using a MACSQuant analyzer in combination with the Flowlogic Software (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany).

Phagocytic activity

Fluorescein-conjugated *S. aureus* BioParticles were incubated with HL60 cells, at a ratio of 50 BioParticles per cell, in 12-well plates for 1 h at 37°C. Each sample was then analyzed for phagocytic abilities using a MACSQuant analyzer in combination with the MACSQuantify software (Miltenyi Biotec GmbH).

Cell surface marker determination

HL60 cells (1.0×10^5 cells) suspended in 0.5% bovine serum albumin in phosphate-buffered saline were stained by incubating them on ice for 20 mins with one of the following monoclonal antibodies or isotype-matched monoclonal antibodies: Percp-eFluor710-labeled anti-CD11b (Thermo Fisher Scientific), FITC-labeled anti-CD14 (Thermo Fisher Scientific), and a combination of unlabeled antibodies to flavocytochrome b_{558} (also known as CYBB; Medical & Biological Laboratories Co., Ltd., Tokyo, Japan) and APC-labeled F(ab')2 antibody (Thermo Fisher Scientific). Then, stained cells were analyzed using a MACSQuant analyzer in combination with the MACSQuantify software (Miltenyi Biotec GmbH) and FlowJo Software (Tree Star, Ashland, USA).

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted using the TRIzol LS reagent according to the manufacturer's instructions. qRT-PCR was performed in 10 μL of iTaq Universal SYBR Green One-Step reaction mixture (Bio-Rad, Richmond, CA, USA) in a CFX96 Real-time System (Bio-Rad). The qRT-PCR protocol was performed in separate tubes: 10 min at 50°C, 40 cycles of denaturation (95°C for 5 min), and annealing (55°C for 30 s) per cycle. The readouts of amplified samples were normalized using those of the amplified ACTB housekeeping gene. Melting curves for each PCR were generated to ensure the purity of the amplification product. The following primer sequences (designed by Bio-Rad) were used: ACTB (NM_001101.3), forward 5′-CGAGAAGATGACCCAGAT-3′ and reverse 5′-GATAGCACAGCCTGGATA-3′; CD11b (NM_001145808.1), forward 5′-GTGGTGAACTGCTCCATC-3′ and reverse 5′-TTATGCGAGGTCTTGATGTAC-3′; and CYBB (NM_000397.3), forward 5′-TTATTCAGTAGCACTCTC-3′ and reverse 5′-ATAATTAATATGAGGCACAG-3′.

Statistical analysis

The results are expressed as the mean ± standard error. One-way analysis of variance, followed by the Tukey-Kramer method, was used to determine the statistical significance of differences between the experimental and control groups. A p-value < 0.05 was considered to be statistically significant.

3. Results

Cell growth and cell cycle

Growth rate of cells treated with genistein or VD_3 alone was not significantly different from that of untreated cells (Fig. 1). Growth rates of VD_3-treated and genistein-treated cells were significantly lower than those of untreated cells on the third day ($p < 0.05$).

As a previous study reported that genistein increases the proportion of G_2/M phases of cell cycles[17], we focused on the cell cycle. No difference was observed in the proportion of cells in the G_2/M phase of the cell cycle between untreated (11.6 ± 1.5%) and genistein-treated (11.5 ± 2.2%) HL60 cells. However, there was a reduction in the proportion of cells treated with VD_3 alone, from 9.5 ± 0.8% to 7.1 ± 0.4%, after adding genistein ($p < 0.05$). Although there was a minimal difference in the proportion of cells in the G_0/G_1 phase between the untreated (34.9 ± 7.0%) and genistein-treated HL60 cells (39.1 ± 2.6%), the proportion significantly increased to 54.5 ± 3.5% in VD_3 treated cells, which further increased to 72.9 ± 2.4% with the addition of genistein ($p < 0.05$) (Table 1). A typical example of our cell cycle results is shown in Fig. 2.

Phagocytic activity

No phagocytic activity was observed in HL60 cells treated with genistein alone or in untreated cells (Fig. 3). With the addition of genistein, the proportion of cells exhibiting phagocytic ability among HL60 cells stimulated by VD_3 significantly increased from 17.8 ± 4.2% to 31.4 ± 3.4% on day 3 ($p < 0.05$).

Cell surface markers and expression of mRNA

We analyzed the expression of antigens against the phagocytic markers CD11b and CYBB and the monocytic marker CD14 in HL60 cells after treatment with genistein, VD_3, or both. After the addition of genistein, the proportion of CD11b-positive cells, among HL60 cells induced to differentiate using VD_3, increased significantly from 34.2 ± 3.1% to 71.1 ± 1.6% and from 66.4 ± 2.4% to 84.4 ± 0.5% on the first and second days, respectively ($p < 0.05$) (Fig. 4A). The proportion of CYBB-positive cells, among VD_3-induced differentiated HL60 cells, increased significantly after

the addition of genistein from 2.3 ± 1.2% to 4.2 ± 1.5% and 13.2 ± 4.8% to 20.8 ± 7.3% on the first and second days, respectively ($p < 0.05$) (Fig. 4B).

The expression of CD14, which is known to be induced by VD_3 later than 24 hours[12], was clearly induced by VD_3 on day 2 and day 3. However, the CD14 expression induced by VD_3 was not further increased by the addition of genistein (Fig. 4C). Genistein alone had no effect on the expression of any marker examined in HL60 cells. Next, we assessed the mRNA expression of CD11b and CYBB, the protein expression levels of which were increased by genistein. In cells stimulated with VD_3, the addition of genistein increased the relative mRNA expression levels of CD11b and CYBB approximately 3.7-fold ($p < 0.05$) and 2.5-fold ($p < 0.05$), respectively, on day 1 (Fig. 5A, 5B).

4. Discussion

In this study, genistein further potentiated the cell proliferation inhibitory effect of VD_3. This observation was supported by the fact that genistein augmented the effect of VD_3 to increase the G_0/G_1 ratio, which is a hallmark of cell differentiation during the cell cycle. Genistein treatment also increased the proportions of phagocytic cells; these findings are concordant with those of previous reports on VD_3-induced monocytic differentiation using HL60 cells[18,19]. These results suggest that genistein promotes the VD_3-induced differentiation of HL60 cells.

According to a study by Katagiri et al.[19], the expression of CD11c (complement receptor 4: CR4) antigen is induced by genistein in VD_3-stimulated HL60 cells. As it has been reported that CD11b (complement receptor 3: CR3) is more important for phagocytosis than CD11c in monocytes[20], we analyzed the effect of genistein on CD11b expression. Genistein further increased the proportion of CD11b-positive cells, which had already been increased by VD_3. Thus, our findings and those of Katagiri et al. suggest that genistein affects an expression mechanism that is shared by CD11b and CD11c during the VD_3-induced differentiation of HL60 cells.

Superoxide, a reactive oxygen species (ROS), is an important factor for innate immune system-mediated killing of microorganisms, such as bacteria; it is produced by Nox2-containing CYBB. In cells stimulated with VD_3, genistein upregulated the expression of the CYBB antigen, which is consistent with previous reports which showed that genistein enhanced production of ROS[18].

Furthermore, genistein upregulated the protein and mRNA expression of CD11b and CYBB in VD_3-stimulated HL60 cells. Therefore, genistein was suggested to be involved in the transcriptional upregulation of genes encoding CD11b and CYBB. However, the mechanism by which genistein promotes these expressions needs to be analyzed in detail in future studies.

CD14 is a monocyte-specific marker and receptor, which, in addition to TLR4, recognizes LPS derived from gram-negative bacteria[6]. The expression of CD14 was not affected by the addition of genistein to VD_3-induced monocytic differentiated HL60 cells. Therefore, there is probably a distinct route by which CD14 expression is induced, which is unaffected by genistein.

The concentration of genistein used in our experiments lies within the serum concentration range achieved via consumption of food and/or supplements (4.3–16.3 μmol/L)[21]. Thus, genistein might have promoted the phagocyte phenotype *in vivo*.

Collectively, our results, combined with a previous report on genistein promoting ATRA-induced neutrophil differentiation[17], suggest that genistein, at dietarily achievable concentrations, can preferentially promote common phenotypes in phagocytes, such as neutrophils and monocytes, but not monocyte-specific ones, in the context of VD_3-induced monocytic differentiation.

Conflicts of interest

The authors declare no conflict of interest.

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Table 1. Cell cycle distribution in HL60 cell cultures treated with each indicated reagent.

Cell-cycle phase	Treatment[1)]			
	None	Gen	VD_3	VD_3 + Gen
G_0/G_1	34.9 ± 7.0	39.1 ± 2.6	54.5 ± 3.5*	72.9 ± 2.4#
S	53.5 ± 8.4	49.4 ± 2.9	36.0 ± 3.5	20.0 ± 2.2
G_2/M	11.6 ± 1.5	11.5 ± 2.2	9.5 ± 0.8	7.1 ± 0.4*

HL60 cells were cultured with or without 10 µmol/L genistein (Gen) and either with or without 100 nmol/L 1α,25-dihydroxyvitamin D_3 (VD_3) for 3 days. Data are expressed as mean ± SEM. The differences between groups were analyzed with one-way analysis of variance (ANOVA), followed by Tukey's test (n = 3); *$p < 0.05$ vs None; # $p < 0.05$ vs VD_3 alone.

[1)] Each column indicates the percentage (%) of total cells in the aforementioned cell-cycle phase.

Figure legends

Fig. 1 Effect of genistein (Gen) on the growth of 1α,25-dihydroxyvitamin D_3 (VD_3)-induced monocytic HL60 cells. HL60 cells were cultured with or without 10 μmol/L Gen and with or without 100 nmol/L VD_3 for 3 days, and cell growth rate was analyzed. The vertical axis indicates the cell growth rate relative to the cell number on day 0. Data are shown as mean ± SEM. The differences between groups were analyzed with one-way analysis of variance (ANOVA), followed by Tukey's test (n = 3); * $p < 0.05$ vs. None.

Fig. 2 A representative example of the cell cycle distribution in HL60 cell cultures treated with each indicated reagent. HL60 cells were cultured with or without 10 μmol/L genistein (Gen) and with or without 100 nmol/L 1α,25-dihydroxyvitamin D_3 (VD_3) for 3 days. The data are representative of three independent experiments, as shown in Table 1.

Fig. 3 Effect of genistein (Gen) on the phagocytic ability of 1α,25-dihydroxyvitamin D_3 (VD_3)-treated HL60 cells. HL60 cells were cultured with or without 10 μmol/L Gen and with or without 100 nmol/L VD_3 for 3 days. *Staphylococcus aureus* cells were incubated with HL60 cells grown in a 12-well plate (50 bacterial cells per HL60 cell) for 1 h at 37°C. Phagocytic ability was assessed using flow cytometry. The vertical axis indicates percentage of phagocytizing cells per total cells. Data are shown as mean ± SEM. The differences between groups were analyzed with one-way (ANOVA), followed by Tukey's test (n = 3); * $p < 0.05$ vs. VD_3.

Fig. 4 Effect of genistein (Gen) on the expression of phagocytic markers CD11b and CYBB and monocytic marker CD14 in 1α,25-dihydroxyvitamin D_3 (VD_3)-treated HL60 cells. HL60 cells were treated with 10 μmol/L Gen, 100 nmol/L VD_3, or 10 μmol/L Gen in combination with 100 nmol/L VD_3. Upper panel: The proportions of A) CD11b-positive, B) CYBB-positive, and C) CD14-positive HL60 cells were determined using flow cytometry. The vertical axis indicates percentage of positive

cells per total cells. A) and B), data are shown for 2 days; C), data are shown for 3 days. Data are shown as mean ± SEM, and differences between groups were analyzed with one-way analysis of variance (ANOVA), followed by Tukey's test; * $p < 0.05$ vs. None; # $p < 0.05$ vs. VD_3. Lower panel: The histograms are representative of three independent experiments.

Fig. 5 Effect of genistein (Gen) on CD11b and CYBB mRNA expression in 1α,25-dihydroxyvitamin D_3 (VD_3)-induced monocytic HL60 cells. HL60 cells were treated with or without 10 μmol/L Gen in the presence of 100 nmol/L VD_3 for 1 day. The relative expression levels of A) CD11b mRNA and B) CYBB mRNA were determined using quantitative real-time polymerase chain reaction. The vertical axis indicates the expression level relative to the expression of VD_3. Data are shown as mean ± SEM. Differences were analyzed using Tukey's test (n = 3); *$p < 0.05$ vs. VD_3 treated groups