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Identification of caffeoylquinic acid derivatives from Brazilian propolis as constituents involved in induction of granulocytic differentiation of HL-60 cells

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Abstract—We have previously reported that Brazilian propolis extracts inhibited growth of HL-60 human myeloid leukemia cells, which is partly attributed to the induction of apoptosis associated with granulocytic differentiation. In this study, we isolated three compounds which induce granulocytic differentiation evaluated by nitroblue tetrazolium (NBT)-reducing assays from the water extract of propolis and identified as 4,5-di-*O*-caffeoylquinic, 3,5-di-*O*-caffeoylquinic, and 3,4-di-*O*-caffeoylquinic acids by NMR analysis. Cell growth inhibitory activity of these caffeoylquinic acids was found in HL-60 cell, which was mainly attributed to the induction of apoptosis. Furthermore, the potency of caffeoylquinic acid derivatives to induce granulocytic differentiation was examined in HL-60 cells. Caffeic, quinic, and chlorogenic acids had no effects on the NBT-reducing activity, while 3,4,5-tri-*O*-caffeoylquinic acid induced more than 30% of NBT-positive cells. These results suggest that the number of the caffeoyl groups bound to quinic acid plays an important role in the potency of the caffeoylquinic acid derivatives to induce granulocytic differentiation. This is the first report demonstrating that the caffeoylquinic acid derivatives induce granulocytic differentiation of HL-60 cells.

1. Introduction

Cell differentiation is essential for normal growth and homeostasis. Some cancer cells including leukemia cells exhibit a defect in their capacity to mature to nonreplicating adult cells, thereby remaining in a highly proliferative state and outgrowing their normal cellular counterparts. The induction of terminal differentiation, leading to the eventual elimination of tumorigenic cells and reestablish normal cellular homeostasis, represents an alternative approach to the treatment of cancer by conventional antineoplastic agents.[1,2]

HL-60 human myeloid leukemia cell line is a promyelocyte which proliferates continuously and is known to differentiate into granulocytes or monocytes by variety of compounds, such as butylate, dimethyl sulfoxide, and vitamin D3.[3,4] In particular, all-*trans*-retinoic acid (ATRA) is a potent inducer of HL-60 cell differentiation into granulocytes and used for clinical therapy for acute promyelocytic leukemia.[5,6] Furthermore, some flavonoids and polyphenol derivatives have been reported to induce HL-60 cell differentiation.[7,8] In these points, we focused on propolis which has been shown to comprise more than 300 constituents, including benzoic acids, flavonoids, and cinnamic acid derivatives.[9,10]

Propolis is a resinous substance collected by honeybees from plant sources and thought to serve a protective role against potential predators. Propolis has been used in folk medicine and has been reported to possess a therapeutic or prophylactic effect against inflammation, heart disease, diabetes mellitus, hepatotoxicity, and cancer.[11,12] In recent years, it has become a common additive in health foods and beverages. We have previously reported that the propolis extracts exerted growth-inhibitory effect on HL-60 cells, which is partly attributed to the induction of apoptosis associated with granulocytic differentiation.[13] A better understanding of ingredients

Keywords: Caffeoylquinic acid derivatives; Differentiation, HL-60; Propolis.
Abbreviations: ATRA, all-*trans* retinoic acid; 3,4-diCQA, 3,4-di-*O*-caffeoylquinic acid; 3,4,5-triCQA, 3,4,5-tri-*O*-caffeoylquinic acid; 3,5-diCQA, 3,5-di-*O*-caffeoylquinic acid; 4,5-diCQA, 4,5-di-*O*-caffeoylquinic acid; NBT, nitro blue tetrazolium; WEP, water extract of propolis.
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in propolis underlying the induction of granulocytic differentiation could provide a useful clue for the development of new candidate drugs and health food additives. In this study, we isolated three compounds involved in the induction of granulocytic differentiation from the water extract of propolis (WEP), and identified as dicaffeoylquinic acids. Furthermore, the potency of caffeoylquinic acid derivatives to induce granulocytic differentiation was elucidated.

2. Results and discussion

2.1. Isolation of compounds having nitroblue tetrazolium (NBT)-reducing activity from WEP

We have previously reported that WEP inhibited HL-60 cell growth, which is partly attributed to apoptosis associated with granulocytic differentiation.[13] In this study, our interest was to identify the constituents involved in the induction of granulocytic differentiation assessed by NBT-reducing potency. For this purpose, we have separated the active constituents from the WEP by using stepwise chromatographies. As a result of the HPLC separation in combination with the NBT-reducing assay, two major (b, c) and one minor (a) peaks were obtained from the Fr. 2b and Fr. 2c (Fig. 1). These peaks were individually collected and identified to be 4,5-di-*O*-caffeoylquinic acid (4,5-diCQA) (peak a) and 3,5-di-*O*-caffeoylquinic acid (3,5-diCQA) (peak b) in Fr. 2b, and 3,4-di-*O*-caffeoylquinic acid (3,4-diCQA) (peak c) in Fr. 2c, by NMR analysis which showed the spectra consistent with those previously reported.[14] The structures of these three compounds are shown in Figure 2, and the proportional contents of 4,5-diCQA, 3,5-diCQA, and 3,4-diCQA contained in WEP were 0.8%, 4.9%, and 6.1%, respectively.[13]

When HL-60 cells were exposed to 4,5-diCQA, 3,5-diCQA, and 3,4-diCQA at concentrations 40, 80, and 120 μM, the cell growth was suppressed in a dose-dependent manner (Fig. 3A). No viable cells were observed when HL-60 cells were treated with over 160 μM of these compounds (data not shown). We then examined their ability to induce granulocytic differentiation of HL-60 cells using the NBT-reduction assay. The treatment with 500 μg/mL WEP induced 11.8% NBT-positive cells, while 4,5-diCQA, 3,5-diCQA, and 3,4-diCQA induced 6.3%–11.3%, 7.0%–11.2%, and 5.3%–10.7%, respectively, in a dose range of 40–120 μM. These three compounds exhibited similar cytotoxic and NBT-reducing activities. Considering much higher contents of 3,5-diCQA and 3,4-diCQA relative to 4,5-diCQA, the former two compounds might be greater in contribution to the overall activity.

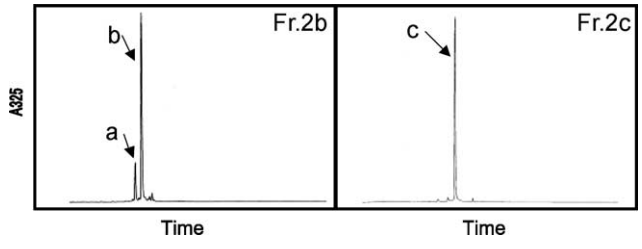

Figure 1. HPLC profile of Fr. 2b and Fr. 2c. Fractions indicated by arrows (a, b, c) were individually collected and analyzed by NMR. They were identified to be 4,5-di-*O*-caffeoylquinic acid (4,5-diCQA), 3,5-di-*O*-caffeoylquinic acid (3,5-diCQA), and 3,4-di-*O*-caffeoylquinic acid (3,4-diCQA), respectively.

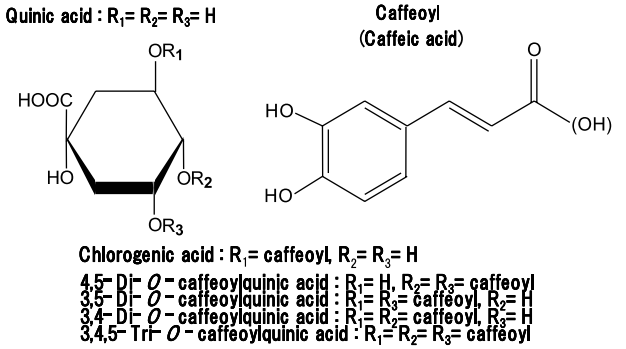

Figure 2. Chemical structures of caffeoylquinic acids.

To examine whether the granulocytic differentiation induced by these dicaffeoylquinic acids leads to apoptosis in HL-60 cells, the morphological observations were made by fluorescence microscopy. When HL-60 cells were treated with 4,5-diCQA, 3,5-diCQA, or 3,4-diCQA for 72 h, we observed the typical morphological features characteristic of apoptosis, such as nuclear condensation and fragmentation in Hoechst 33342-stained HL-60 cells. More than 15% apoptotic cells were observed when HL-60 cells were treated with 80 μM of 4,5-diCQA, 3,5-diCQA, or 3,4-diCQA (Table 1). Figure 4 showed typical apoptic cells treated with 3,4-diCQA, and similar morphological changes were also observed in HL-60 cells treated with 4,5-diCQA or 3,5-diCQA (data not shown). These results suggest that these dicaffeoylquinic acids inhibit cell growth, which is partly attributed to the granulocytic differentiation leading to apoptosis.

2.2. Effects of caffeoylquinic acid derivatives in propolis on cell growth and granulocytic differentiation in HL-60 cells

Besides 4,5-diCQA, 3,5-diCQA, and 3,4-diCQA, propolis contains caffeoylquinic acid derivatives such as monocaffeoylquinic acid (chlorogenic acid)[10,13] and 3,4,5-tri-*O*-caffeoylquinic acid (3,4,5-triCQA)[15] (Fig. 2). Chlorogenic acid, diCQAs, and triCQA are esters of quinic acid and 1, 2, and 3 caffeic acids (Fig. 2). Yoshimoto et al.[16] isolated several kinds of caffeoylquinic acid derivatives from sweet potato leaf and showed their antimutagenic activity, which is 3,4,5-triCQA > 4,5-diCQA = 3,5-diCQA = 3,4-diCQA > chlorogenic acid in this order. Matsui et al.[15] isolated caffeoylquinic acid derivatives from propolis as constituents involved in α-glucosidase inhibitory activity and demonstrated that 3,4,5-triCQA has the most potent activity among them including their moieties (quinic and caffeic acids). These studies suggest that an increasing number of caffeoyl groups promotes the potential functions of caffeoylquinic acid derivatives.

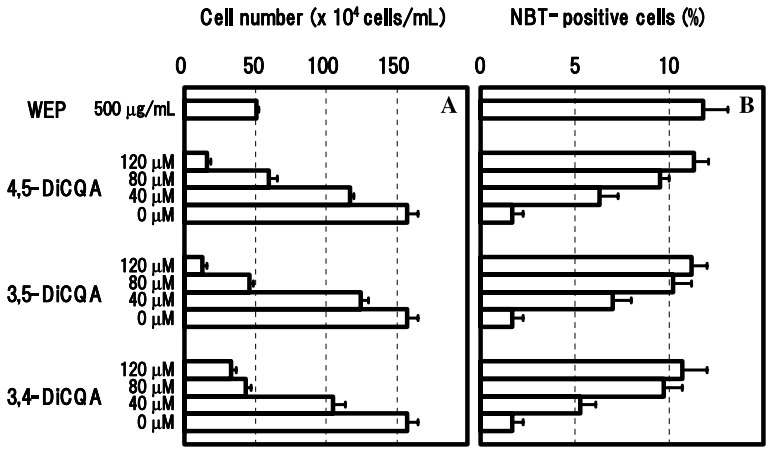

Figure 3. Effects of dicaffeoylquinic acids on the cell growth (A) and NBT-reducing activity (B). HL-60 cells were treated with dicaffeoylquinic acids (4,5-diCQA, 3,5-diCQA, and 3,4-diCQA) for 72 h at indicated concentrations. Living cells were counted by the Trypan Blue exclusion method, and NBT-reducing activities were determined and shown as the percentage of NBT-positive cells in 200 cells. The mean values with standard deviations from three experiments were shown.

Table 1. Percentage of apoptic cells

Caffeoylquinic acid	Concentration	Apoptic cells (%)
WEP	500 μg/mL	15.5 ± 1.8
Control	—	4.0 ± 0.5
4,5-diCQA	80 μM	17.8 ± 1.6
3,5-diCQA	80 μM	17.2 ± 0.8
3,4-diCQA	80 μM	18.7 ± 1.4
3,4,5-triCQA	120 μM	17.5 ± 1.5

HL-60 cells were treated with water extract of propolis (WEP) or each caffeoylquinic acid for 72 h and stained with Hoechst 33342. Typical apoptic cells showing nuclear condensation and fragmentation were counted in 200 cells by a fluorescence microscope. The mean values with standard deviations from three experiments were shown.

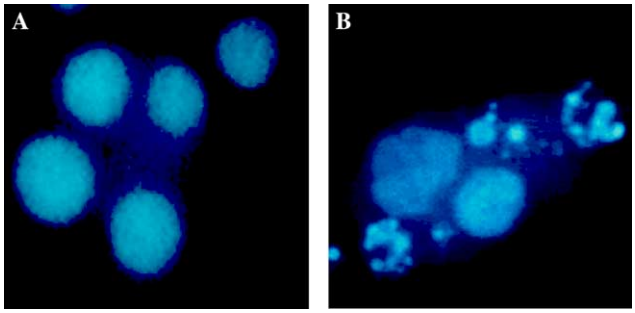

Figure 4. Microscopic observations of HL-60 cells after the treatment with dicaffeoylquinic acid (3,4-diCQA). HL-60 cells were treated with 80 μM of 3,4-diCQA for 72 h, stained with Hoechst 33342, and observed by the fluorescence microscopy. (A) Control and (B) 3,4-diCQA-treated cells.

In this context, besides 4,5-diCQA, 3,5-diCQA, and 3,4-diCQA, we have examined the effects of caffeoylquinic acid derivatives (quinic, caffeic, and chlorogenic acids and 3,4,5-triCQA) on the growth and granulocytic differentiation of HL-60 cells.

3,4,5-TriCQA, which was found to be contained at very small level in WEP, inhibited the cell growth of HL-60 and induced NBT-reducing ability in a dose-dependent manner (Fig. 5). Maximal response was obtained with a concentration of 160 μM, which produced NBT-positive cells in 32% of viable cells, equivalent to approximately threefold potent than 4,5-diCQA, 3,5-diCQA, and 3,4-diCQA. Typical morphological changes characteristic of apoptosis were observed in 3,4,5-triCQA-treated cells (Table 1), suggesting that 3,4,5-triCQA also inhibited HL-60 cell growth, partly due to the granulocytic differentiation leading to apoptosis. Chlorogenic and caffeic acids also inhibited the growth of HL-60 cells in which no apoptotic characters were observed, but did not induce the marked NBT-reducing activity (Fig. 5). Caffeic acid had marked growth-inhibitory activity at a concentration of 16 μM, which may be direct cytotoxicity mediated via its antioxidative activity.[16] When HL-60 cells were treated with quinic acid, neither growth-inhibitory nor NBT-reducing activities were observed (Fig. 5). Thus, these results lead us to assume that there would be some relationships between the number of caffeoyl groups bound to quinic acid and the potency to induce differentiation in HL-60 cells.

In the current study, we isolated 4,5-diCQA, 3,5-diCQA, and 3,4-diCQA from WEP as constituents involved in the induction of granulocytic differentiation of HL-60 cells. Furthermore, 3,4,5-triCQA was also shown to be a potent inducer for granulocytic differentiation among caffeoylquinic acid derivatives. This is the first report describing the granulocytic differentiation-inducing activity of the caffeoylquinic acid derivatives. We have previously reported that, besides WEP, the ethanol extract of propolis has the ability to induce granulocytic differentiation of HL-60 cells. Thus, 3,4,5-triCQA could be one of the constituents in the ethanol extract of propolis, because this compound was found to be more abundant in the ethanol extract than WEP (data not shown). The granulocytes are the most numerous and most important cellular component in the innate immune response. Caffeoylquinic acid derivatives have been reported to enhance spreading and mobility of macrophages, which precede macrophage infiltration

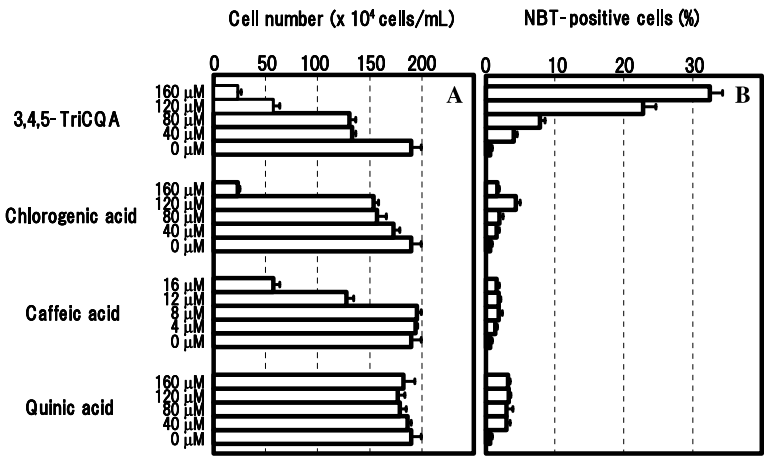

Figure 5. Effects of caffeoylquinic acid derivatives on the cell growth (A) and NBT-reducing activity (B). HL-60 cells were treated with chlorogenic acid, 3,4,5-triCQA, quinic acid, or caffeic acid at indicated concentrations. After 72 h, living cells were counted by the Trypan Blue exclusion method, and NBT-reducing activities were determined and shown as the percentage of NBT-positive cells in 200 HL-60 cells. The mean values with standard deviations from three experiments were shown.

into tissues affected by injury or infection.[14] Further understanding of caffeoylquinic acid derivatives underlying the effects on immune response as well as on cancer cells would provide a scientific basis for the better therapeutic application or dietary supplement.

3. Experimental section

3.1. Materials

Brazilian propolis was collected in Minas Gerais state.[13] Five volumes of water was added to 50 g of propolis and stirred for 4 h at 45 °C. Following the removal of insolubles by centrifugation, the supernatant was freeze–dried. The resultant powder (10 g) was designated as WEP. 3,4,5-TriCQA was isolated from propolis as described previously.[15] NBT, phorbol 12-myristate 13-acetate, and ATRA were purchased from Sigma–Aldrich (St. Louis, MO, USA); chlorogenic acid was from Nacalai Tesque (Kyoto, Japan); caffeic acid (3,4-dihydroxycinnamic acid) and D-(−)-quinic acid (hexahydro-1,3,4,5-tetrahydroxy-benzoic acid) were from Tokyo Kasei Kogyo (Tokyo, Japan).

3.2. Isolation of active compounds from WEP

WEP was dissolved in water, applied to a column (70 × 400 mm) of ODS (Fuji Silysia Chemical, Aichi, Japan) equilibrated with 25% methanol and successively eluted with 1000 mL of 25% methanol, 3000 mL of 50% methanol, and 1500 mL of 99% methanol. Each fraction denoted as Fr. 1, Fr. 2, and Fr. 3 for eluate with 25% methanol, 50% methanol, and 99% methanol, respectively, was taken to dryness. The NBT-reducing activity was detected in Fr. 2. Fr. 2 was dissolved in a solvent containing 98% acetonitrile and 2% acetic acid and applied to an HPLC system (Model PU-980 and UV-970; JASCO, Japan) with a Capcell Pak ACR (Shiseido, Tokyo, Japan) C18 column (20 × 250 mm), and the column was eluted 1000 mL of the same solvent. The three fractions denoted as Fr. 2a, Fr. 2b, and Fr. 2c were collected to dryness to assay for the NBT-reducing activity. The activity was detected in Fr. 2b and Fr. 2c. Each active fraction was dissolved in 98% acetonitrile and 2% acetic acid and applied on an HPLC system (Model 600S and 486, Waters) with a Capcell Pak AG-120 (Shiseido) C18 column (4.6 × 250 mm). The mobile phase consisted of 1% acetic acid in water (A) and 1% acetic acid in acetonitrile (B), and the column was eluted using a gradient of 10% B (0–10 min), 10–15% B (10–25 min), 15–75% B (25–80 min), and 70% (80–90 min) at a flow rate of 1.0 mL/min. As a result of detection at a wavelength of 325 nm, two major and one minor peaks were obtained from the Fr. 2b and Fr. 2c, respectively (Fig. 1). Each fraction was applied again to an HPLC system with a Capcell Pak ACR with the same conditions described above, and the observed peaks were individually collected.

3.3. Cell culture and NBT assay

HL-60 cells were purchased from Health Science Research Resources Bank (Osaka, Japan) and maintained in RPMI-1640 (Sigma) containing 10% heat-inactivated FBS (Sigma) at 37 °C in a 5% CO_2 atmosphere. For cell growth and differentiation assays, cells were seeded in 12-well plates at a density of 200,000 cells/2 mL/well and incubated with each sample. Samples were dissolved in water and used in a 1:100 dilution in the medium. As a control, cells were also cultured with water alone. Cell number was counted by the Trypan Blue exclusion method at 3 days after the treatments. NBT-reducing activity was determined by the method described previously as a marker of granulocytic differentiation.[17] In each experiment, we always confirmed when HL-60 cells were exposed to 1–10 μM ATRA, NBT-positive cells were observed in over 70% of total cell population (data not shown), which is consistent with a previous report.[18]

3.4. NMR

^{1}H NMR and ^{13}C NMR and H-H COSY, HSQC, and HMBC spectra were recorded with the MERCURYplus

300NB NMR instrument (Varian, Palo Alto, CA, USA). The solvent used was deuterium methanol-d_4. Tetramethylsilane was used as an internal standard.

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