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Inflammation Research

Regulation of cell proliferation, inflammatory cytokine production and calcium mobilization in primary human T lymphocytes by emodin from *Polygonum hypoleucum Ohwi*

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Abstract: *Objective and Design:* This study was designed to elucidate action mechanisms of four anthraquinones identified from *Polygonum hypoleucum Ohwi* (*P. hypoleucum Ohwi*) on primary human T lymphocytes.
Material and methods: The cells were isolated from peripheral blood.
Treatment: T cells were treated with 5 to 60 μM of four anthraquinones with or without phytohemagglutinin (PHA; 5 μg/ml) for 3 days. Effects of 4 anthraquinones on T lymphocyte proliferation, production and gene expression of inflammatory cytokines and intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) were determined. Data were assessed with Student's *t*-test.
Results: On a percentage basis, emodin had the highest suppressing activity on T lymphocyte proliferation with an IC_{50} of 11.2 ± 0.6 μM. Emodin decreased cytokine production, IL-2 mRNA expression, and $[Ca^{2+}]_i$ in activated T cells.
Conclusions: We hypothesize that the inhibitory mechanisms of emodin on activated T cells proliferation are related to the impairment of cytokine production, IL-2 mRNA level and $[Ca^{2+}]_i$ in the cells.

Key words: Emodin – T Lymphocytes – Proliferation – Inflammatory cytokines – Ca^{2+}

Introduction

Polygonum hypoleucum Ohwi (*P. hypoleucum Ohwi*) is a Chinese herb that has been used for the treatment of arthritis [1]. In the previous studies, we have found that *P. hypoleucum Ohwi* is a growth modulator for tumor cells and human mesangial cells [2, 3]. Although this plant has been utilized in Chinese herbal medicinal prescriptions to improve tissue inflammation for a long time, there has been relatively scarce definitive evidence to prove its immunopharmacological activity. In order to identify the active ingredients in *P. hypoleucum Ohwi* that are responsible for its possible clinical effects, pure compounds of this plant were evaluated in immune responses of primary human T lymphocytes.

An intense inflammatory process is a characteristic pathologic feature of arthritis [4, 5]. In these patients there is an accumulation of polymorphonuclear neutrophils, macrophages, and T cells in the synovial tissue and synovial fluid [6–9]. There is now convincing evidence that cytokines secreted by T cells or other immune cells such as interleukin-4 (IL-4), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ) play a role in arthritis [10–15]. Both IL-6 and TNF-α serve as chemotactic factors for various leukocyte population and are important inflammatory factors. The production of cytokines such as interleukin-2 (IL-2) and IL-4 is pivotal in the growth of T lymphocytes induced by antigens or phytohemagglutinin (PHA) [16]. In patients with arthritis, the levels of inflammatory cells, T cells and cytokines have been shown to be significantly elevated in the synovial tissue or synovial fluid, suggesting a possible pathological role for these cells and substances [17]. One of the therapeutic objectives in arthritis is to reduce the local inflammatory response by diminishing the inflammatory cell activation and proliferation, as well as the inflammatory cytokine production. The blockade of T lymphocyte activation and proliferation and cytokines production is one of the main antiinflammatory mechanisms [18].

In this investigation, four anthraquinones emodin, emodin 1-O-β-D-glucoside (49A), physcion (62A), and physcion 1-O-β-D-glucoside (50A) were identified from *P. hypoleucum Ohwi*. The primary human T lymphocytes were isolated from peripheral blood and used as target cells. The effects of emodin, 49A, 50A, and 62A on primary human T lymphocyte proliferation and production and gene expression of cytokines were determined. It is now clear that interaction of human T cells with PHA induces increase in $[Ca^{2+}]_i$, which is

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an important early event in the initiation of T cell activation and proliferation [19]. We determined the actions of emodin on intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in primary human T lymphocytes induced by PHA.

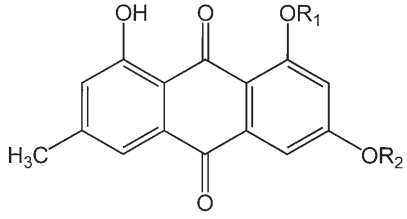
Materials and methods

Plant Material

The stems of *P. hypoleucum Ohwi* were purchased from the Chinese medicine shop in Taipei. The plant was identified by Mr. Jun-Chih Ou, resident medicinal plant expert of The National Research Institute of Chinese Medicine. A reference sample (NRICM 81001) was retained at the Institute.

Emodin and other anthraquinones isolated from P. hypoleucum Ohwi

The stems of *P. hypoleucum Ohwi* were air-dried and cut into small pieces before grinding. The ground stem (200 gm) was then extracted with methanol at 50 °C for 24 h. The solvent was removed under reduced pressure and the residue mixed with distilled water (100 ml). The mixture solution was extracted with n-butanol (3 × 100 ml) and the solvent then evaporated to yield the crude extract (2.3 gm). The sample, absorbed on silica gel (sample/adsorbent (v/v) = 1/8), was subjected to dry flash column chromatography. Sufficient hexane was passed through the column to expel all of the air. Extensive gradient elution were then employed using hexane (250 ml), chloroform (300 ml) and 15% chloroform/methanol (v/v, 200 ml) to yield 75 fractions. The like fractions were combined to give four main fractions with monitoring by thin-layer chromatography (TLC) and the solvent was removed under reduced pressure. Each combined fraction was further purified by rechromatography and recrystallization. Emodin (68 mg) was obtained from the second combined fractions as orange-red solid and recrystallized from chloroform/methanol, mp 202–205 °C. Emodin 1-O-β-D-glucoside (49A; 37 mg) was obtained from the fourth combined fractions and recrystallized from acetone/methanol, mp 192–197 °C. Physcion (62A; 27 mg) was obtained from the first combined fractions as orange solid and recrystallized from chloroform/acetone, mp 202–205 °C. Physcion 1-O-β-D-glucoside (50A; 54 mg) was obtained from the third combined fractions and recrystallized from acetone/methanol, mp 250–254 °C. Their structures are shown in Fig. 1. The NMR data of 4 anthraquinones were compatible with the previously reported data [2]. The purity of all pure compounds isolated from *P. hypoleucum Ohwi* was assessed by an HPLC purity program (reverse column RP-18). The purity of emodin, 49A, 50A, and 62A was estimated to be over 98% based on the clear spectral data and sharp melting point. They were dissolved in dimethylsulfoxide (DMSO) and then stored at 4 °C until use.



(A) Emodin $R_1=R_2=H$

(B) Physcion $R_1=H$, $R_2=CH_3$

(C) Emodin 1-O-β-D-glucoside R_1=β-D-glucoside, $R_2=H$

(D) Physcion 1-O-β-D-glucoside R_1=β-D-glucoside, $R_2=CH_3$

Fig. 1. The structures of emodin, 49A, 50A, and 62A purified from *P. hypoleucum Ohwi*.

General methods and apparatuses for compound purification

Melting points were measured on a micro melting point hot-stage apparatus and were uncorrected. ^{1}H- and ^{13}C-NMR spectra were taken on Brucker AM-300 WB spectrometer in DMSO-*d*6 with tetramethyl silane (TMS) as an internal standard operating at 300 MHz for ^{1}H- and 75 MHz for ^{13}C-NMR. Dry flash column chromatography was performed on silica gel (230–400 mesh, Merck). TLC was carried out on precoated kiesel gel 60 F_{254} (silica gel plated, 0.25 mm thick, Merck) plates; spots were visualized under UV light (254 and 365 nm) irradiation and by spraying with 10% molybdatophosphoric acid solution followed by heating at 120 °C.

Human subjects

Twelve healthy male subjects (22 to 36 yr, mean age 27 yr) were chosen for this investigation. The experimental protocol had been reviewed and approved by the institutional human experimentation committee. Written informed consent was obtained from each and every subject.

Preparation of primary human T lymphocytes

Heparinized human peripheral blood (80 ml) was obtained from normal healthy volunteers. Human mononuclear cells were isolated by the Ficoll-Hypaque gradient density method as described previously [20]. After depletion of adherent cells on plastic dishes, T lymphocytes were isolated by erythrocyte rosetting. The erythrocyte rosette positive fraction contained < 5% monocytes or B lymphocytes, as assessed by flow cytometric analysis. T cells were resuspended to a concentration of 2×10^6 cells/ml in RPMI-1640 medium (GIBCO), supplemented with 2% heat-inactivated fetal calf serum (FCS; Hyclone), 100 U/ml penicillin and 100 μg/ml streptomycin.

Lymphoproliferation test

The lymphoproliferation test was modified from previously described [20]. The density of T lymphocytes was adjusted to 2×10^6 cells/ml before use. 100 μl of cell suspension was divided into each well of a 96-well flat-bottomed plate (Nunc 167008, Nunclon, Raskilde, Denmark) with or without 5 μg/ml PHA (Sigma). Cyclosporin A was used as a positive control [21]. Emodin, 49A, 62A, or 50A was added to the cells at 5, 10, 15, 30 or 60 μmol/L. The plates were incubated in 5% CO_2-air humidified atmosphere at 37 °C for 3 days. Subsequently, tritiated thymidine (1 μCi/well, NEN) was added into each well. After a 16 h incubation, the cells were harvested on glass fiber filters by an automatic harvester (Dynatech, Multimash 2000, Billingshurst, UK). Radioactivity in the filters was measured by a scintillation counting. The inhibitory activities of four anthraquinones on T lymphocytes proliferation were calculated by the following formula:

$$\text{Inhibitory Activity (\%)} = \frac{\text{Control Group (CPM)} - \text{Experiment group (CPM)}}{\text{Control group (CPM)}} \times 100$$

Determination of cytokines production in primary human T lymphocytes

T lymphocytes (2×10^5 cells/well) were cultured with PHA alone or in combination with 5, 10, 15, 30 or 60 μmol/L of emodin, 49A, 62A, or 50A for 3 days. The cell supernatants were then collected and assayed for IL-2, IL-4, IL-6, TNF-α, and IFN-γ concentrations by the enzyme immunoassays, respectively (EIA; R&D systems, Minneapolis, USA). No detectable cross-reactivity with other cytokines has been reported for the EIA kits used.

Extraction of total cellular RNA

The total cellular RNA was extracted from T lymphocytes by a method described previously [22]. The 5×10^6 cells were activated with or without PHA and cocultured with 60 µmol/L of emodin for 18 h. T cells were collected and washed by cold Tris-saline containing 25 mmol/L Tris, pH 7.4, 130 mmol/L NaCl, 5 mmol/L KCl and then suspended in NDD buffer containing 1% NP-40, 0.5% sodium deoxycholate, and 1% dextran sulphate. After centrifugation, the supernatants were extracted with a phenol-chloroform mixture. The extracted RNA was precipitated with 100% cold ethanol. The total cellular RNA was pelleted by centrifugation and redissolved in diethyl pyrocarbonate (DEPC)-treated H_2O. The concentration of the extracted RNA was calculated by measuring the optical density at 260 nm. The ratio of the optical density at 260 nm to that at 280 nm was always higher than 1.8. The quality of RNA was assessed by the intactness of 28S and 18S bands and lack of degradation on agarose-gel electrophoresis.

Synthesis of first strand complementary deoxyribonucleic acid

Aliquots of 1 µg of RNA were reverse-transcribed using the Advantage™ RT-for-PCR kit from CLONTECH according to the manufacturer's instructions. Briefly, 1 µg RNA in 12.5 µl of DEPC-treated H_2O was mixed with 20 µmol/L of oligodeoxythymidine (oligo $dT)_{18}$, and heated at 70 °C for 10 min, then quick-chilled on ice. The following reagents were added to the tube: 6.5 µl of concentrated synthesis buffer (50 mmol/L Tris-HCl, pH 8.3; 75 mmol/L KCl; 3 mmol/L $MgCl_2$; 0.5 mmol/L dNTPs; and 0.5 unit RNase inhibitor), and 200 U of the Moloney murine leukemia virus (MMLV) reverse transcriptase. The reaction was initially incubated at 42 °C for 1 h, and then at 94 °C for 5 min to terminate the reaction. The tube was added with 80 µl of DEPC-treated H_2O and then stored at −20 °C for use in the polymerase chain reaction (PCR).

PCR

The PCR was performed in an air thermocycler according to the manufacturer's instructions as described previously [23]. Briefly, 10 µl of the first-strand complementary deoxyribonucleic acid (cDNA) was mixed with 0.75 µmol/L primers, 4 units of Taq polymerase, 10 µl of reaction buffer (2 mmol/L Tris-HCl, pH 8.0; 0.01 mmol/L ethylenediaminetetraacetate, EDTA; 0.1 mmol/L Dithiothreitol, DDT; 0.1% Triton X-100; 5% Glycerol; and 1.5 mmol/L $MgCl_2$), and 25 µl of water in a total volume of 50 µl. All primer pairs for the cytokines were designed from the published human cDNA sequence data [24–28] (Table 1). The PCR was done at the following setting of the air thermocycler: denaturing temperature of 94 °C for 1 min, annealing temperature of 60 °C for 1 min and elongation temperature of 72 °C for 80 s for the first 40 cycles and finally elongation temperature of 72 °C for 10 min. Following the reaction, the amplified product was taken out of the tubes and run on 2% agarose gel.

The effect of emodin on $[Ca^{2+}]_i$ of primary human T lymphocytes

The $[Ca^{2+}]$i of primary human T lymphocytes was determined as described previously by Grynkiewics et al. [29]. The primary human T lymphocytes were loaded with 5 µmol/L fura-2-acetoxymethyl ester (fura-2-AM) at 37 °C for 30 min. The cells were then resuspended in Ca^{2+}-free RPMI-1640 medium without phenol red to a concentration of 4×10^6 cells/ml. In each experiment, 0.5 ml of cells suspension was equilibrated with an equal volume of 2 mmol/L Ca^{2+}-containing medium at 37 °C. The cells were added with 2.5 µl of 0.1% DMSO or emodin (15 µmol/L or 30 µmol/L) at the 30th sec then stimulated with 2.5 µl of PHA (5 µg/ml) and the changes in fluorescence with time were recorded. During the measurement, the cell suspension was kept at 37 °C and continuously stirred. The fluorescent activity was recorded at Ex = 340, 380 nm and Em = 505 nm using an F-4500 fluorescence spectrophotometer (Hitachi) with a multi-wavelength time-scan program.

Determination of cell viability

Approximately 2×10^5 primary human T lymphocytes were activated with or without PHA and cocultured with 0.1% DMSO, or 60 µmol/L of emodin, 49A, 50A, or 62A for 4 days. Total, viable, and non-viable cell numbers were counted under the microscope with the help of a hemocytometer following trypan blue staining. The dead cells had a blue color. The percentage of viable cells was calculated:

$$\text{Viability (\%)} = \frac{\text{Viable Cell Number}}{\text{Total Cell Number}} \times 100$$

Statistical analysis

Data were presented as Mean ± SD and the differences between groups were assessed with Student's *t*-test.

Results

Effects of 4 anthraquinones on primary T lymphocytes proliferation

The results indicated that treatment with PHA for 3 days stimulated cell proliferation by about 9 fold (1500 ± 704 vs.

Table 1. Nucleotide sequences of the primers used for amplification of cytokines in primary human T lymphocytes.

Cytokine	Sequence	Predicted size (bp)
IL-2	5′: GTC ACA AAC AGT GCA CCT AC 3′: GAA AGT GAA TTC TGG GTC CC	262
IL-4	5′: CGG CAA CTT TGA CCA CGG ACA CAA GTG CG 3′: AGG ACA CTT CCT TCG GTT GGT CTC ATG CA	244
IL-6	5′: TCA ATG AGG AGA CTT GCC TG 3′: CGT CCT GAT CTG TTG AGT AG	260
TNF-α	5′: ACA AGC CTG TAG CCC ATG TT 3′: TCA GAC CCG TCC AGA TGA AA	427
IFN-γ	5′: TCT GCA TCG TTT TGG GTT CTC 3′: CTC TAC TGA AGC TTT TCG ACT	320
β-Actin	5′: TTG AGA CCT TCA ACA CCC 3′: CTC TAC TGA AGC TTT TCG ACT	1300

13 500 ± 1242 CPM, $P < 0.001$) as reflected by the increase in tritiated thymidine uptake. Neither the tritiated thymidine uptake in the resting nor stimulated states (1679 ± 169 vs. 1500 ± 704 CPM; 14 179 ± 592 vs. 13 500 ± 1242 CPM) were affected by DMSO treatment. To study the effects on primary human T lymphocyte proliferation, resting or PHA activated cells were treated with 5, 10, 15, 30, and 60 µmol/L of emodin, 49A, 50A, or 62A, respectively. As shown in Figure 2C, 2D, and 2E, 49A, 50A, and 62A had little effect on proliferation in either resting or PHA activated cells. By contrast, emodin blocked the cell proliferation activated by PHA. As shown in Fig. 2B, while emodin had little effect on tritiated thymidine uptake in primary human T lymphocytes, the enhanced uptake observable in activated cells was significantly suppressed by emodin. Furthermore, the inhibitory effect of emodin on the activated cells was concentration-dependent. At 5 µmol/L, the inhibitory percentage of emodin was 26.5 ± 6.5%. The corresponding degree of inhibition for 60 µmol/L was 89.3 ± 4.7% and it was comparable to that of 12.5 µmol/L cyclosporin A. Moreover, the IC_{50} of emodin on activated primary human T lymphocytes proliferation was 11.2 ± 0.6 µM (Table 2).

The viability of activated primary human T lymphoctyes treated with 4 anthraquinones

To elucidate the suppressant mechanism of emodin on T cells proliferation, whether it was related to direct cytotoxicity, the viability of activated T lymphocytes treated with or without

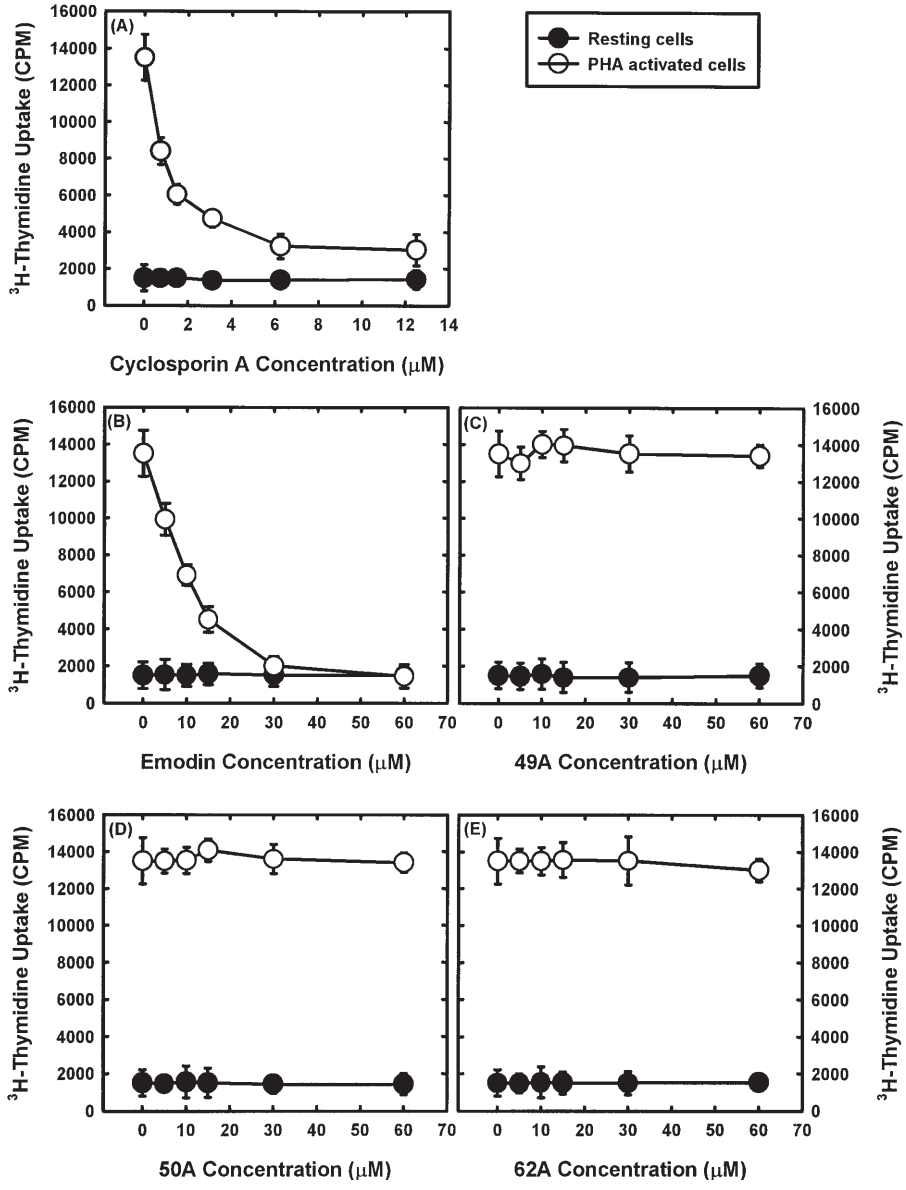


Fig. 2. The inhibitory activity of emodin on T lymphocyte proliferation. T cells (2×10^5/well) were treated with or without PHA (5 µg/ml) in the presence or absence of various concentrations of emodin, 49A, 50A, or 62A for 3 days. Cyclosporin A was added to the cells at 0.75, 1.5, 3.125, 6.25, and 12.5 µmol/L. The proliferation of cells was detected by tritiated thymidine uptake (1 µCi/well). After a 16 h incubation, the cells were harvested by an automatic harvester then radioactivity was measured by a scintillation counting. Each point represents the mean of three independent experiments.

Table 2. IC_{50s} of 4 anthraquinones on activated primary human T lymphocyte proliferation and cytokine production.

	IC_{50} (μM)			
	Emodin	49A	50A	62A
Cell proliferation	11.2 ± 0.6	> 60	> 60	> 60
IL-2 production	17.0 ± 2.0	> 60	> 60	> 60
IL-4 production	10.3 ± 1.6	> 60	> 60	> 60
IL-6 production	12.7 ± 1.3	> 60	> 60	> 60
TNF-α production	10.0 ± 2.1	> 60	> 60	> 60
IFN-γ production	9.1 ± 0.8	> 60	> 60	> 60

Primary human T lymphocytes (2×10^5 cells/well) were activated PHA (5 μg/ml) and cocultured with various concentration of emodin, 49A, 50A, and 62A for 3 days. The cell proliferation was determined by tritiated thymidine uptake. The supernatants were collected and cytokine concentration determined by EIA. The IC_{50s} were calculated as inhibition 50% of cell proliferation or cytokine production.

60 μmol/L of 4 anthraquinones was determined. As shown in Fig. 3, emodin, 49A, 50A, and 62A had no cytotoxicity because the viability of cells were not significantly decreased after being treated with the drugs for 4 days. We suggest that the inhibitory actions of emodin on T cell proliferation induced by PHA are not through direct cytotoxicity.

Effects of 4 anthraquinones on cytokine production in primary human T lymphocytes

To further delineate whether the impairment of activated primary human T lymphocytes proliferation was related to cytokine production, the cells were activated with or without PHA in the presence or absence of emodin, 49A, 50A, or 62A for 3 days. Supernatants were then collected and IL-2, IL-4, IL-6, TNF-α, and IFN-γ productions were assayed by EIA. The results are shown in Fig. 4. The IL-2, IL-4, IL-6, TNF-α, and IFN-γ productions in primary human T lymphocytes were induced by PHA. While 49A, 50A, and 62A had no effects, the stimulated production of IL-2, IL-4, IL-6, TNF-α, and IFN-γ productions in activated T cells was significantly decreased by emodin. Furthermore, the inhibitory activities of emodin on this cytokine production were concentration-dependent. At 60 μmol/L, the stimulated production of IL-2, IL-4, IL-6, TNF-α, and IFN-γ in activated T cells was completely blocked by emodin, their concentrations returning to almost the same as those produced in resting cells. The IC_{50} of emodin on primary human T lymphocytes IL-2, IL-4, IL-6, TNF-α, and IFN-γ production were listed in Table 2.

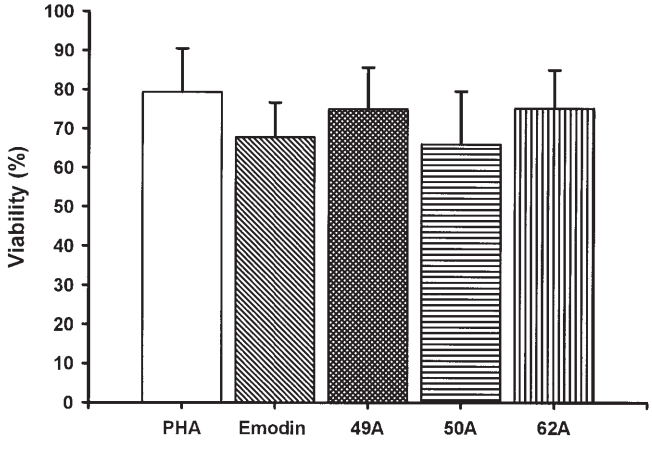


Fig. 3. The viability of activated primary human T lymphocytes treated with 4 anthraquinones. 2×10^5 primary human T lymphocytes were cultured with PHA (5 μg/ml) in the presence or absence of medium (control), 0.1% DMSO, or 60 μM of emodin, 49A, 50A, or 62A for 4 days. The total, viable, and non-viable cell number were counted after staining by trypan blue and cell viability was calculated. Each bar represents the mean of three independent experiments.

Effects of emodin on cytokine mRNA expression in primary human T lymphocytes

To study whether emodin impeded cytokine production in T cells through inhibition of cytokine gene expression, total cellular RNA was extracted from T lymphocytes in the presence or absence of 60 μmol/L emodin and available for RT-PCR. Initially, we examined the dose-response relationship of the PCR-amplification of cDNA (data not shown). The exponential phase of amplification was determined by performing for 20, 30, 40, and 50 cycles. We found that 40 cycles of PCR were optimal for all the cytokines (data not shown). The results of RT-PCR analyses are shown in Fig. 5A. The mRNA for β-actin was detectable in the samples treated with PHA (Lane 1) and PHA and emodin (Lane 2), respectively. It indicated that emodin did not affect β-actin mRNA expression in T lymphocytes. Levels of IL-4, IL-6, TNF-α, and IFN-γ mRNA expression in T cells were unchanged by emodin. In contrast, PCR products for IL-2, amplified from PHA treated T cell RNA preparations were decreased by emodin. Furthermore, the results of the laser densitometry analysis are shown in Fig. 5B. It demonstrated that the ratio of IL-2 mRNA to β-actin mRNA in PHA activated T cells was significantly decreased by emodin.

Effect of emodin on $[Ca^{2+}]_i$ in activated primary human T lymphocytes

To study whether the impairment of proliferation in activated T lymphocytes was related to $[Ca^{2+}]_i$, the cells were incubated with or without the emodin, and $[Ca^{2+}]_i$ in cells was determined. The results are shown in Fig. 6. As shown in curve A, when primary human T lymphocytes were incubated with DMSO at the 30th sec and PHA was added at the 180th sec, $[Ca^{2+}]_i$ began to increase around at the 200th sec and reached maximal aprox. at the 250th sec (curve A). The increase in $[Ca^{2+}]_i$ was no different in DMSO treated to untreated T cells (data not shown). By contrast, when emodin was added at the 30th sec, the increase in $[Ca^{2+}]_i$ induced by PHA was decreased in a dose dependent manner (curve B). However, 49A, 50A, and 62A did not suppress intracellular free Ca^{2+} concentration of primary human T lymphocytes (data not shown).

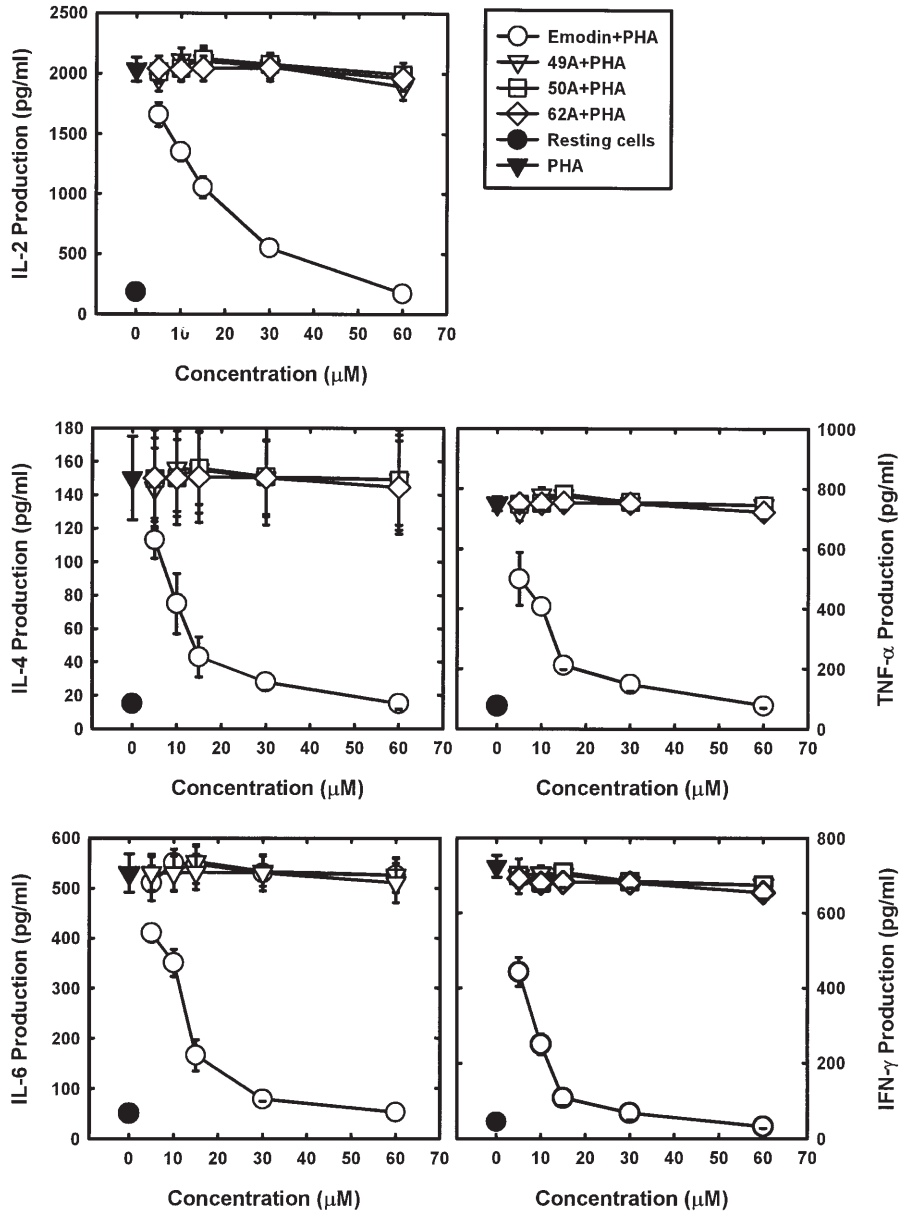


Fig. 4. Cytokine production in primary human T lymphoctyes treated with emodin, 49A, 50A, or 62A. T cells (2×10^5/well) were treated by 0, 5, 10, 15, 30, and 60 μM of emodin, 49A, 50A, or 62A with or without PHA (5 μg/ml) for 3 days. Then the cell supernatants were collected and IL-2, IL-4, IL-6, TNF-α, and IFN-γ concentration was determined by EIA, respectively. Each point is the mean of three independent experiments.

Discussion

In the present study, action mechanisms of four anthraquinones, emodin, emodin 1-O-β-D-glucoside, physcion, and physcion 1-O-β-D-glucoside isolated from *P. hypoleucum Ohwi* on primary human T lymphocyte proliferation activated with PHA were defined. Results demonstrated that a maximal increase in DNA synthesis induced by PHA was inhibited by emodin. The growth-suppressive actions of emodin were not explained by a drug-induced reduction in cell viability. We observed that emodin decreased production of several cytokines in activated T cells. The levels of IL-2 mRNA in T cells stimulated with PHA were reduced by emodin. In addition, $[Ca^{2+}]_i$ in activated primary human T lymphocytes was attenuated by emodin. We suggest that emodin interferes with some regulatory events required for PHA activated T cells proliferation.

Emodin (1,3,8-trihydroxy-6-methylanthraquinone) is an orange-red crystalline compound [30]. It is an anthraquinone and produced by many plants including Ex. *Rheum officinale*, *Aloe barbadensis*, *Rhamnus frongula*, and *Polygonum cuspidatum* [31]. Microorganisms, especially fungi Ex. *Penicillium islandicum Sopp*, and *Aspergillus glaucus* also synthesize these anthraquinones [32, 33]. We have identified it from *P. hypoleucum Ohwi* [2]. The biological activities of emodin include: (a) inhibition of envelope viral replication [34, 35], (b) reduction of interleukin-1 (IL-1), IL-2, and IL-2 receptor expression in human mononuclear cells [36], (c)

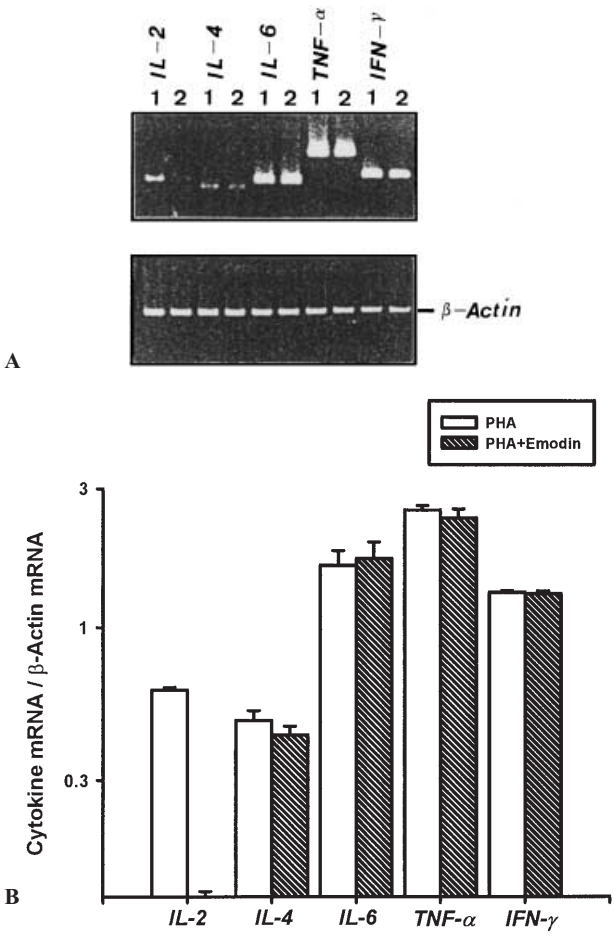


Fig. 5. Effects of emodin on cytokine mRNA expression in T cells detected by RT-PCR analysis. 5×10^6 T cells activated with PHA (5 μg/ml) in the presence or absence of 30 μM emodin for 18 h. The total cellular RNA was isolated from T cells treated with PHA (Lane 1) emodin and PHA (Lane 2), respectively. Aliquots of 1 μg of RNA were reverse-transcribed for synthesis of first strand cDNA. Briefly, 10 μl of the first-strand cDNA was applied for the PCR test. The PCR was done as described in Materials and Methods. Following the reaction, the amplified product was taken out of the tubes and run on 2% agarose gel (A). Graphical representation of laser densitometry of various cytokines expression in PHA stimulated T cells in the presence or absence of emodin. Each band was quantitated using laser scanning densitometer SLR-2D/1D (Biomed Instruments Inc., Fullerton, CA, USA). The ratio of each cytokine mRNA to β-actin mRNA was calculated. Each bar is the mean of three independent experiments (B).

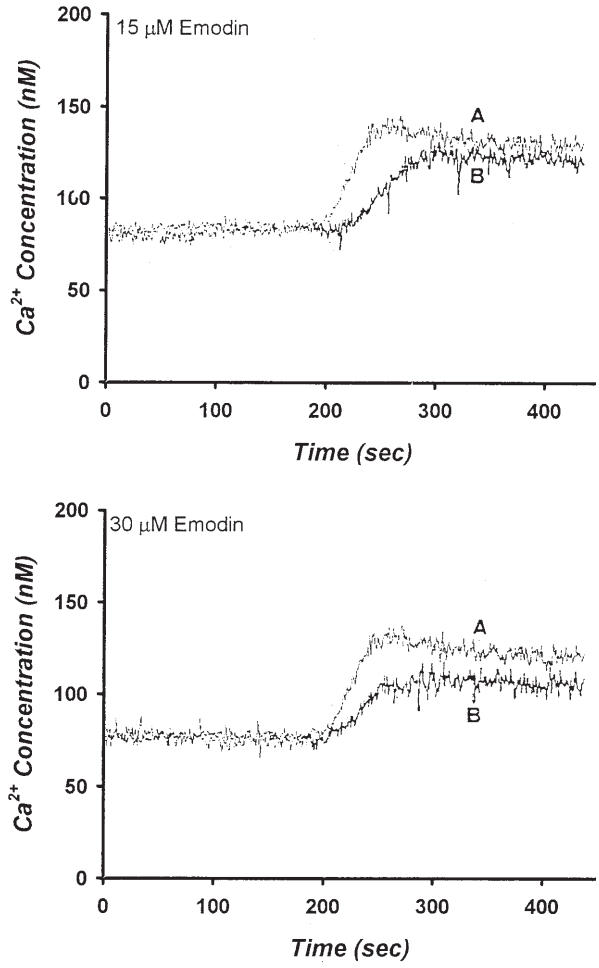


Fig. 6. The effect of emodin on $[Ca^{2+}]_i$ induced in PHA treated primary human T lymphocytes. The primary human T lymphocytes were loaded with 1 μM fura-2-AM at 37 °C for 30 min. The cells were then resuspended in RPMI-1640 medium without phenol red to a concentration of 4×10^6 cells/ml. In each experiment, 0.5 ml of equilibrated T cell suspension was added with 2.5 μl of DMSO (0.1%; curve A) or emodin (15 or 30 μM; curve B) at the 30th sec then stimulated with 2.5 μl of PHA (5 μg/ml) at the 180th sec and the changes in fluorescence with time recorded. The fluorescent activity was recorded by an F-4500 fluorescence spectrophotometer (Hitachi) with multi-wavelength time scan program.

inhibition of protein tyrosine kinase activity [37–39], (d) inhibition of adenosine 3′-5′-cyclic monophosphate (cAMP) phosphodiesterase (PDE) activity [40], (e) enhancement of the formation of Topo II-DNA cleavable complexes related to antitumor activity [41, 42], (f) free radical scavenging activity [43], (g) modulation of Ca^{2+} influx in skeletal muscle [44], and (h) inhibition of inflammation in rat liver tissue [45]. Thus, it appears that emodin may possess many biological activities including immunomodulatory function. In the present study, the results indicate that emodin was a growth inhibitor for primary human T lymphocytes.

49A is a glycosylated emodin. The glucose was added at C1 position (R1=β-D-glucoside). When the –OH group of emodin was displaced by glucose, the inhibitory activity of emodin on activated primary human T lymphocytes was blocked. When the C3 position of emodin was changed to a methyl group (R2=CH_3) and termed physcion (62A), 62A lost suppressant activity. When 62A was glycosylated at C1 position (R1=β-D-glucoside; 50A), the inhibitory activity of the compound on primary human T lymphocytes was decreased. These results indicate that both C1 and C3 position of emodin were important for this inhibitory function. It is unlikely that emodin suppressed primary human T lymphocyte proliferation through cytotoxic effects because no cell death was noticed after primary human T lymphocytes were treated with emodin. The possible inhibitory effect of DMSO on primary human T lymphocytes was also studied in these experiments. Primary human T lymphocyte proliferation, $[Ca^{2+}]_i$ and cell viability were not changed by DMSO. There-

fore, the inhibitory functions of emodin on the cells were unlikely related to DMSO. The morphology and characteristics of primary human T lymphocytes treated with emodin, 49A, 50A, or 62A were similar, suggesting that the inhibitory action of emodin was not related to the pH, osmolarity, or other physiology variables in different preparations (data not shown).

The results of emodin impairment cytokine production in primary human T lymphocytes were compatible with data reported by Huang et al. [36], which indicate IL-2 production is impeded in emodin-treated human mononuclear cells. Interaction of T cells with antigens or mitogens such as PHA initiates a cascade of biochemical events and a series of genes expression, which are included in a carefully controlled order as the cells pass through G0, G1, and S phase and then proliferation. For example, transition from G0 to G1 is marked by transcriptional activation of the IL-2 receptor and IL-2 genes (and in some cases IL-4). Subsequent G1 events and initiation of DNA synthesis are dependent on induction of IL-2 receptor and on a supply of IL-2 from autocrine or external supply. Although the molecular mechanisms involved in regulating passage through the cell cycle in T cells stimulated with PHA remain largely unknown, growth modulators or other external events that affect the T cell proliferation are ultimately likely to act by controlling the expression or function of the products of these genes [46]. In the present study, the purity of primary human T cells was 97%. Although we could not exclude the possibility that B cells or monocytes might be involved in these studies, Chenug et al. proved that following addition of PHA to T cells for 1 h, sufficient to induce proliferation of T cells, approximately 75% to 90% of the response was seen of cells incubated with PHA for the entire 72 h culture period [46]. IL-2 could be detected in the culture supernatants 24 h later. When comparing PHA-induced changes in $[Ca^{2+}]_i$, IL-2 secretion and tritiated thymidine incorporation, there was a very clear correlation between these responses. Thus, PHA is used as a T cell mitogen in many studies. On the other hand, Jurkat cells, a human T cell leukemia cell line, were used as target cells and effects of emodin on IL-2 production in the cell cultures were determined. The preliminary results indicated that emodin inhibited IL-2 production in Jurkat cells. We suggest that effects of emodin on primary human T lymphocytes may not be direct via antigen presenting cells.

We proved that IL-2, IL-4, IL-6, TNF-α, and IFN-γ production in T cells activated with PHA was decreased by emodin. Results of RT-PCR indicated that with the exception of IL-2 mRNA, the levels of other cytokine mRNAs in activated T cells were not decreased by emodin and the action mechanisms may be through: (1) blocking cytokine secretory pathways of the cells or (2) reducing total cell numbers of activated T cells. Although we did not determine intracellular cytokine levels in activated T cells, IL-4, IL-6, TNF-α and IFN-γ production was calculated in picrogram per cell (cytokine production/total cell numbers). The preliminary results showed that cytokine production by each cell (pg/cell) was not different in activated T cell cultures treated with or without emodin. It indicated that these cytokines could be secreted from activated T cells. In addition, viabilities of emodin-treated and untreated T cells were not different, but total cell numbers of activated T cells treated with emodin were significantly lower than those of the untreated cells. Thus, we predict that the impairment of IL-4, IL-6, TNF-α and IFN-γ production in activated T cells treated with emodin may be due to a decrease in the proliferating cell numbers. By contrast, the decreasing of IL-2 production was related to emodin suppressing IL-2 mRNA levels in primary human T lymphocytes. These actions are similar to those of cyclosporin A, which induces the arrest of T cells proliferation by inhibiting IL-2 transcription [21]. IL-2 plays an important role in the growth of T lymphocytes [16]. Thus, we conclude that the inhibitory effects of emodin on T cell proliferation induced by PHA are related to cytokine production and IL-2 mRNA level in the cells. Furthermore, a decrease in IL-2 mRNA level could be due to a decrease of gene transcription or mRNA stability. We did not determine whether emodin affected the stability of IL-2 mRNA. However, we understand that the Ca^{2+}-dependent pathways participate in and are required for the transcriptional regulation of the IL-2 gene. Gelfand et al. proved that the decrease in intracellular Ca^{2+} concentration would induce impairment of IL-2 production in T lymphocytes [47]. In the present study, we found that emodin decreaed $[Ca^{2+}]_i$ in primary human T lymphocytes induced by PHA. We predict that emodin causes attenuation of IL-2 mRNA level in activated primary human T lymphocytes, which may be related to impairment of $[Ca^{2+}]_i$ in the cells. On the other hand, Miyokawa et al. reported that emodin increases cellular cAMP concentration through inhibition of PDE activity, then reduces $[Ca^{2+}]_i$ in treated cells [40]. Recently, the effect of emodin on cAMP concentration in T cells was determined in a preliminary study. The results indicated that emodin could increase cAMP concentration in T cells activated with PHA (data not shown). We predict that the suppressant mechanism of emodin on $[Ca^{2+}]_i$ in activated T cells may be related to cAMP concentration. However, its detailed mechanisms of action on Ca^{2+} mobilization and IL-2 mRNA transcription in PHA activated T lymphocytes is a subject for further study.

From the present results, we hypothesize that the inhibitory mechanisms of emodin on activated primary human T lymphocytes proliferation, at least in part, are related to: (a) the impairment of $[Ca^{2+}]_i$ in the cells, (b) the attenuation of IL-2 mRNA level in the cells, (c) decrease in cytokine production including IL-2 and IL-4 in the cells, and then (d) inhibition of proliferation in primary human T lymphocytes stimulated with PHA. Taken together, the results of the present study suggest that emodin may also have acted as an antiinflammatory agent, in part, by inhibiting T cell proliferation and cytokine production. These results are compatible with putative pharmacological activities of *P. hypoleucum Ohwi* for treatment of arthritis. Future experiments with treatment of animals with arthritic disease with emodin will be necessary to define whether emodin can reduce experimental arthritic injury. Moreover, this study not only demonstrates that Chinese herbs are potential therapeutic drugs for arthritis but also support a model for future protocol design in preclinical studies.

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