Emodin induces apoptosis of concanavalin A-stimulated murine splenocytes

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Abstract Emodin, one of the major compounds in the herb *Reynoutria elliptica*, is known to maintain immunosuppressive, anti-allergic, anti-cancer, and anti-inflammatory effects. In this study, we assessed the possibility of using emodin to induce apoptosis in stimulated immune cells *in vitro*. After treatment with emodin and concanavalin A (Con A), we observed DNA damage-induced apoptosis in splenocytes. Moreover, treatment with emodin and Con A increased the number of apoptotic splenocytes compared with untreated controls. Emodin also diminished the size of CD45R/B220⁺ cells, CD19⁺CD69⁺ cells, and cDC populations. These results indicate that emodin-induced apoptosis was involved in attenuating the immune activity promoted by DNA damage and in decreasing the number of CD45R/B220⁺ B cells and CD19⁺CD69⁺ activating B cells. This demonstration of emodin inducing apoptosis of Con A-stimulated immune cells indicates its potential utility as a therapy for diseases caused by abnormally activated immune cells.

Keywords: emodin, apoptosis, autoimmune disease, concanavalin A, immune cells

Introduction

Apoptosis is crucial in maintaining homeostasis in multicellular organisms, and changing the apoptotic threshold of immune cells may alter the pathologic progression of some cancers, viral infections, and neurodegenerative disorders. In particular, for autoimmune diseases such as multiple sclerosis, systemic lupus erythematosus, and rheumatoid arthritis, apoptosis of autoreactive immune cells may eliminate or neutralize these destructive entities, thereby preventing or modulating the disease process (1). Observations of several animal models have provided a rich source of information about the potential of apoptosis in alleviating autoimmune diseases. For example, T cell apoptosis in the central nervous system (CNS) of rats with acute experimental autoimmune encephalomyelitis has been suggested to contribute to the spontaneous recovery from disease and development of tolerance (2). Additionally, a low level of lymphocyte apoptosis was found in patients who suffered from the autoimmune diseases rheumatoid arthritis and primary Sjögren's syndrome (3). These results indicate the potential of developing therapeutic strategies that selectively suppress abnormally activated immune cells.

Natural compounds are known to have applications for

inflammatory diseases, often reliant on their low toxicity profile. One such compound is emodin (1,3,8-trihydroxy-6-methylanthraquinone), a major compound in the three herbal families: *Fabaceae* (*Cassia spp.*), *Polygonaceae* (*Rheum*, *Rumex*, and *Polygonum spp.*), and *Rhamnaceae* (*Rhamnus* and *Ventilago spp.*) (4). Therefore, we explored emodin for its documented properties of immune suppressive (5,6), anti-allergic (7,8), anti-cancer (9-11) and anti-inflammatory effects (12,13). Moreover, an apoptotic effect of emodin on human T cells was recently reported (14), implying that it might adjust immune reactions as a dual modulator (15) depending on the activation degree of immune cells. However, whether emodin can selectively induce apoptosis in activated lymphocytes has not been studied.

Concanavalin A (Con A) is a lectin (carbohydrate-binding protein) that binds to cell-surface glycoproteins, including the T cell receptor (TCR), and has been used extensively to study T cell activation. Although Con A is generally known to activate T cells, some research indicates that humoral factors from activated T cells cause a B cell response to Con A (16-18). In the present study, we investigated the pro-apoptotic effect of emodin on Con A-stimulated immune cells to determine whether emodin could induce splenocytes apoptosis and provide therapeutic potential for immune disorders.

Materials and Methods

Plant material Emodin, 6-methyl-1,3,8-trihydroxyanthraquinone, was isolated from the ethyl acetate fraction of the methanol extract of *Polygoni cuspidati* (*P. cuspidati*) radix by Dr. Hyeun Wook Chang (College of Pharmacy, Yeungnam University, Korea) (8). The emodin we used in this study showed a single spot on thin layer chromatography (TLC) plates and 99.5% purity by high-performance liquid chromatography analysis (19). Emodin was prepared by dissolving dimethyl sulfoxide (DMSO) to make stock solutions of 10 mM, and it was further diluted with RPMI-1640 (Gibco-BRL, Life Technologies, Carlsbad, CA, USA) to achieve the desired concentration.

Preparation of splenocytes Cells were isolated from the spleens of C57BL/6 mice (4 mice/group) and prepared as single-cell suspensions. Red blood cells were then lysed with ammonium chloride potassium (ACK) lysis buffer for 10 min at room temperature and washed with Dulbecco's phosphate-buffered saline (PBS, Gibco-BRL). Subsequently, the splenocytes were counted via trypan blue dye (Sigma-Aldrich, St. Louis, MO, USA) exclusion. Finally, these splenocytes (viability >90%) were suspended in an RPMI-1640 medium, 10% fetal bovine serum (Gibco BRL), and 1% antibiotics (100 U/mL penicillin–streptomycin, Gibco BRL).

MTT assay The survival ability of splenocytes treated with emodin and Con A (5 µg/mL) was evaluated in a thiazolyl blue tetrazolium bromide (MTT, Sigma-Aldrich) colorimetric assay in which yellow tetrazolium bromide was converted to its purple formazan derivative via mitochondrial succinate dehydrogenase in viable cells. Moreover, 15-µL MTT (5 mg/mL) was appended to each well, incubated for 72 h at 37°C under 5% CO₂, and then kept for 4 h before appraising the reaction. Afterward, formazan crystals were dissolved in 100 µL of solubilization buffer (pH=4.7), and the absorbance at 570 nm was examined using a plate reader with absorbance at 630 nm as the reference. The absorbance in the untreated wells was regarded as 100% viability.

³H-thymidine incorporation assay The effect of emodin on splenocyte proliferation was assessed using a ³H-thymidine incorporation assay (Amersham, Arlington Heights, IL, USA). Splenocytes (4×10⁵) were incubated with emodin (10 µM) with or without Con A (5 µg/mL) for 72 h at 37°C, 95% humidity, and 5% CO₂; 1 µCi of ³H-thymidine (specific activity=42 Ci/mmol) was added to each well after incubation and allowed to react for 18 h. Cells were then harvested onto glass fiber filters (Amersham) using an automatic cell harvester, and the extent of radioactivity incorporated into their DNA was examined via a liquid scintillation spectrometer (MicroBetaTriLux, Perkin Elmer, Waltham, MA, USA).

DAPI staining To assess the apoptosis of splenocytes by emodin (10 µM) combined with Con A (5 µg/mL), we used 4,6-diamidino-2-

phenylindole, dihydrochloride (DAPI) staining. In general, cultured cells induced to undergo apoptosis exhibit characteristic signs within 6–12 h. For clear detection of the apoptotic population, splenocytes were incubated for 6 h and 12 h after treatment with emodin and/or Con A and were then stained with 1 µg/mL DAPI solution for 10 min in a dark room. Morphological changes in apoptotic cells, including apoptotic body composition with divided nuclei and chromatin condensation, were observed using a fluorescence microscope (Leica, Wetzlar, Germany), and the number of apoptotic cells was evaluated as the ratio of the number of apoptotic cells to that of total splenocytes (400–500 cells).

Comet assay The apoptotic influence of emodin with Con A (5 µg/mL) on the splenocytes was assessed using an alkaline comet assay. Splenocytes were immediately treated with emodin (10 µM) and Con A (5 µg/mL) incubated for 6 or 12 h. The basic alkaline technique was performed as described in Kang *et al.* (20). Slides were viewed under the fluorescence microscope and an image analyzer (Komet 5.5, Kinetic Imaging, Liverpool, UK). Comet tail DNA, tail length, and olive tail movement were measured as the distance from the core of the cell nucleus to the apex of the tail in pixel units. A tail DNA%, which represents the extent of DNA damage, was examined as the mean length of 100 tails—%tail DNA=tail DNA/(head DNA+tail DNA)×100.

Annexin V/7-AAD assay The Annexin V/7-Aminoactinomycin D (7-AAD) assay was performed according to the manufacturer's protocol using a Phycoerythrin (PE) Annexin V Apoptosis Detection Kit (BD Pharmingen, San Jose, CA, USA). Splenocytes treated in duplicate with emodin (10 µM) with or without Con A (5 µg/mL) were harvested after 6 h. Then cells were washed twice with cold PBS and resuspended in 1× binding buffer at a concentration of 1×10⁶ cells/mL. Next, 5 µL of PE Annexin V and 5 µL 7-AAD were added after transferring 100 µL of the solution (1×10⁵ cells) into a 5-mL culture tube. Later, cells were gently vortexed and incubated for 15 min at room temperature (25°C) in the dark. Eventually, 400 µL of 1× binding buffer was added to each tube, and the Annexin V-PE and 7-AAD positive cells were analyzed using a BD fluorescence-activated cell sorting (FACS) Calibur™ flow cytometer and the FlowJo software (FlowJo, Ashland, OR, USA).

Surface staining and intracellular cytokine staining Specific cell populations stimulated by emodin with Con A (5 µg/mL) were identified using FACS, which rapidly and concurrently measures diverse characteristics of single cells. Single-cell suspensions of splenocytes (1×10⁶/well) were treated in duplicate with emodin (0, 10, and 20 µM) and Con A (5 µg/mL) and harvested after incubation for 6 h. Then cells were stained for 15 min at 4°C with one of the following fluorescent tags: anti-CD4-FITC (H129.19), anti-CD8-PE (53–6.7), anti-CD19-FITC (1D3), anti-CD45R/B220-PE (RA3-6B2), anti-CD11c-FITC (HL3), anti-IL-17-PE (TC11-18H10), anti-IL-4-PE (11B11),

anti-IL-10-PE (JES5-16E3), anti-IgG-PE (MOPC-21) (BD Pharmingen), and anti-CD69-PE (H1.2F3) (eBioscience, San Diego, CA, USA). Finally, intracellular flow cytometry for cytokines, IL-17, IL-4, IL-10, and IgG, was performed by using Cytofix/Cytoperm (BD Biosciences, San Jose, CA, USA), as per the manufacturer's instructions. All samples were assessed by using a BD FACS Calibur™ flow cytometer (BD Biosciences) and FlowJo software (FlowJo).

Enzyme-linked immunosorbent assay (ELISA) ELISA was performed to identify the effects of emodin on the production of cytokines. Single-cell suspensions of splenocytes (1×10^7 /well) were incubated with emodin (0 and 10 μM) and Con A (5 $\mu\text{g}/\text{mL}$) for 6 h, and supernatants were collected from the cell suspensions. These supernatants were analyzed according to the manufacturer's recommendations using mouse cytokine-specific ELISA kits (Biolegend, San Diego, USA).

Statistical analysis Results are presented as means $\pm$ standard deviation and represent three independent experiments. Data were assessed using one-way analysis of variance (ANOVA) and $p < 0.05$ was regarded as statistically significant.

Results and Discussion

Emodin suppresses the proliferation of Con A-stimulated splenocytes

An MTT assay was performed to establish the survival ability of splenocytes incubated for 72 h in the presence of emodin with or without Con A (5 $\mu\text{g}/\text{mL}$). As shown in Fig. 1A, none of the emodin concentrations applied (2.5, 5, 10, and 20 μM) was cytotoxic for splenocytes. However, compared to the control recipients of Con A only, emodin with Con A decreased splenocytes' survival at specific concentrations, i.e., 10 and 20 μM emodin with Con A significantly decreased the number of surviving cells (Fig. 1B, $p < 0.05$). On the basis of this finding, we selected 10 μM as the emodin dose for additional experiments. Also, treatment with emodin and Con A suppressed the proliferation of splenocytes at all concentrations tested in the ^3H -thymidine incorporation assay. In particular, emodin (2.5, 5, 10, and 20 μM) combined with Con A groups significantly decreased such proliferation (Fig. 1C, $p < 0.05$). These results indicate that although emodin alone was not cytotoxic to splenocytes, emodin with Con A decreased the number of surviving cells and suppressed the proliferation of those that remained.

Emodin induces apoptosis in Con A-stimulated splenocytes

The nuclear morphology of splenocytes treated with emodin plus Con A was assessed to determine whether evidence of apoptosis was present. In Fig. 2B and 2C, arrows point to nuclei undergoing apoptosis as splenocytes manifest significant condensation and fragmentation upon treatment with emodin plus Con A. Apoptotic fragments in emodin plus Con A-treated splenocyte groups (6 h (Fig.

Fig. 1. Emodin with or without Con A (5 $\mu\text{g}/\text{mL}$) influenced the survival of splenocytes. (A, B) Viable cells were evaluated via the MTT assay. (C) The proliferation of splenocytes was estimated via ^3H -thymidine incorporation. All experiments were conducted in triplicate, and data points are expressed as means $\pm$ S.D. Statistical evaluation of the experimental groups compared with their corresponding control groups. * $p < 0.05$, *** $p < 0.005$

2B) and 12 h (Fig. 2C)) increased more in comparison to the Con A control group (6 h: $13.2 \pm 1\%$, 12 h: $17.4 \pm 1.5\%$, control: 0%; Fig. 2A and 2D, $p < 0.001$).

Next, DNA damage due to emodin/Con A treatment of splenocytes was identified using the alkaline comet assay. As the characteristics of splenocytes in Con A activated control (Fig. 3A) and those under Con A activated emodin-treatment (Fig. 3B (6 h) and 3C (12 h)) reveal, comet parameters significantly increased in emodin-treated splenocytes. The respective percentage of tail DNA in Con A activated emodin-treated splenocytes was significantly higher than that of Con A activated nontreated controls (6 h: $29.4 \pm 1.3\%$, 12 h: $33.5 \pm 1.7\%$, control: $3.2 \pm 0.5\%$; Fig. 5D, $p < 0.001$). Similarly, tail movement (6 h: $98.3 \pm 2.8 \mu\text{m}$, 12 h: $112.0 \pm 4.9 \mu\text{m}$, control: 32.7 ± 0.5

Fig. 2. DAPI staining of cells treated with 10 μ M emodin after (A) 0, (B) 6, and (C) 12 h incubation. Also shown is (D) the rate of apoptotic cell death. The experiments were conducted in triplicate, and data points are expressed as mean $\pm$ SD. Statistical evaluation of the experimental groups compared with their corresponding control groups. $p<0.005$

Fig. 3. DNA damage in Con A (5 μ g/mL) and emodin (10 μ M)-treated splenocytes after (A) 0, (B) 6, (C) 12 h incubation was examined using Comet assays. Also shown are (D) percentage of tail DNA, (E) tail length, and (F) olive tail movement expressed in mean $\pm$ SD. for triplicate experiments. Statistical evaluation of the experimental groups compared with their corresponding control groups ($p<0.005$).

μ m; Fig. 5E, $p<0.001$) and tail length (6 h: 16.3 ± 1.5 μ m, 12 h: 24.9 ± 1.2 μ m, control: 1.0 ± 0.1 μ m; Fig. 5F, $p<0.001$) also significantly increased.

Finally, to evaluate the extent of apoptosis after emodin/Con A-treatment, splenocytes were stained with Annexin V/7-AAD. We classified the cellular group that was doubly positive for Annexin V/7-

AAD as late-stage apoptotic splenocytes, whereas those undergoing early-stage apoptosis were characteristically positive for Annexin alone (Fig. 4A). Moreover, the number of late-stage apoptotic splenocytes treated with emodin plus Con A increased to the level of statistical significance (Fig. 4B), clearly indicating that only the combination of emodin with Con A caused apoptosis of splenocytes.

Fig. 4. Apoptosis of splenocytes after treatment with emodin (10 and 20 µM) and Con A (5 µg/mL) was detected via annexin V/7-AAD staining. (A) Comparisons were made with concentrations of emodin (0, 10, and 20 µM) and Con A (5 µg/mL) after 6 h incubation. (B) The apoptotic rate during incubation for 6 h. Statistical evaluation of the experimental groups compared with their corresponding control groups ($p < 0.05$).

Emodin decreases the population of CD45R/B220⁺ cells, CD19⁺CD69⁺ cells, and cDC The number of CD4⁺, CD8⁺, CD19⁺, CD69⁺, CD45R/B220⁺, and CD11c⁺ splenocytes was quantified using flow cytometry to define the effect of emodin (10 and 20 µM) with Con A on specific populations of splenocytes. The CD4⁺ and CD8⁺ cells showed no difference (Fig. 5A). However, the CD45R/B220⁺ (10 µM: 44.0±6.4%, 20 µM: 35.9±1.1%, control: 56.0±3.7%; Fig. 5B, $p < 0.05$), and CD19⁺CD69⁺ (10 µM: 53.3±1.3%, 20 µM: 42.1±1.8%, control: 52.0±5.9%; Fig. 5C, $p < 0.05$) cell populations were significantly lower than those in Con A (5 µg/mL)-treated controls in high concentration (20 µM) of emodin. Furthermore, the conventional dendritic cell (cDC; CD11b⁺ and CD11c^{high}) population was also reduced, whereas total DC and plasmacytoid DC (pDC; CD11b^{low}, CD11c^{high}) displayed no significant change (data not shown). These results suggest that emodin with Con A influenced the immune reaction by inhibiting the proliferation and differentiation of specific B cell and cDC participants.

Several reports have ascribed to emodin biological activities, e.g., antimitosis of activated T cells (14,15). However, some others reports suggest that emodin can suppress the proliferation of mitogen-activated lymphocytes (5,12) and either enhance or suppress immune responses in accordance with the activated state of immune cells as a dual immunomodulator (15). Moreover, emodin may modulate the ratio of the Th1/Th2 paradigm and administer immunosuppressive actions by inducing apoptosis of T cells (5,16). Herein, emodin induced apoptosis of Con A-stimulated immune cells and decreased the number of Con A-stimulated pan and activating B cells; conversely, Con A-stimulated T cells showed no significant changes except for a decreased number of Th 17 cells.

Emodin decreases the IL-17 and IL-10-producing cells To evaluate the suppressive effect of emodin in terms of functional changes in Con A-stimulated splenocytes, we performed the ELISA assay and intracellular staining for cytokines of interest. As depicted in Fig. 6A, the production of IL-17 and IL-10 showed a marked decrease in emodin/Con A treatment of splenocytes ($p < 0.05$). In addition, the population of IL-17-producing cells gated in CD4⁺ cells decreased (10 µM: 1.0±0.2%, control: 2.1±0.6%; Fig. 6B, $p < 0.05$) whereas that of IL-4-producing cells showed no notable change (data not shown). Also, the rate of IL-10-producing cells in CD11c⁺ gated cells significantly diminished (10 µM: 29.5±0.7%, control: 40.4±1.5%; Fig. 6C, $p < 0.05$); however, the reduction of IgG-producing cells was not significant (data not shown). These results suggest that emodin with Con A induced a suppression of cytokine-producing T cells and DCs.

The number of IL-10-producing CD11c⁺ cells significantly decreased as the number of cDCs reduced; however, pDCs and total DCs showed no significant change. Generally, the overall population of DCs is divided into conventional DCs (cDCs) and plasmacytoid DCs (pDCs). Whereas pDCs present antigen and stimulate T cells, cDCs regulate the balance between immunity and tolerance. According to the present results, under conditions of Con A-stimulated immune cells, we presume that the numerical reduction of cDCs and IL-10-producing CD11c⁺ cells was caused by apoptosis and that the outcome might be destruction of the balance in lymphocytes' immune reactions (17-19,21). Here, apoptosis was also involved in attenuating immune activity, precipitating a decrease in the number of pan B cells (CD45R/B220⁺) and activating B cells (CD19⁺CD69⁺). Furthermore, emodin indirectly induced the Con A-stimulation of T

Fig. 5. Influence of emodin (10 and 20 μM) with Con A (5 $\mu\text{g}/\text{mL}$) on the populations of CD4⁺ and CD8⁺ cells was evaluated using a surface-flow cytometry assay. (A) Comparisons were made with concentrations of emodin (0, 10, and 20 μM) and Con A (5 $\mu\text{g}/\text{mL}$) after 6 h of incubation. (A) The percentage of CD4⁺ T cells and CD8⁺ T cells, the effect of emodin (10 and 20 μM) with Con A (5 $\mu\text{g}/\text{mL}$) on (B) B220⁺, and (C) CD19⁺CD69⁺ cells were evaluated using the surface-flow cytometry assay. Comparisons were made with concentrations of emodin (0, 10, and 20 μM) and Con A (5 $\mu\text{g}/\text{mL}$) after 6 h of incubation, and the percentage of the respective cells is shown. Statistical evaluation of the experimental groups compared with their corresponding control groups ($p < 0.05$).

cells because the number of IL-17-producing CD4⁺ T cells was reduced even though CD4⁺ cells, CD8⁺ cells, IL-4-producing CD4⁺ T cells, and IgG⁺-producing CD19⁺ cells showed no difference in comparison to the controls given Con A alone. These results indicate that emodin-induced apoptosis was involved in attenuating DNA damage-promoted immune activity and precipitated the decreased number

of CD45R/B220⁺ B cells, CD19⁺CD69⁺ activating B cells, and IL-17-producing CD4⁺ T cells. Considering that IL-17-producing T cells and autoreactive B cells play a central role in exacerbating autoimmune inflammatory disease, our data support the possibility that emodin may serve as a new therapeutic agent for autoimmune diseases.

In conclusion, our results suggest that emodin induces selective

Fig. 6. Effect of emodin (10 μ M) with Con A (5 μ g/mL) on cytokine production of immune cells was evaluated using ELISA and the intracellular flow cytometry assay. Emodin treatments with 0 and 10 μ M concentrations in the presence of Con A (5 μ g/mL) were compared with each other after 6 h of incubation. (A) The production of cytokines IL-17 and IL-10 measured using ELISA. The percentage of (B) IL-17⁺ cells in CD4⁺ gated cells and (C) IL-10⁺ cells in CD11c⁺ gated cells was measured using flow cytometry. Statistical evaluation of the experimental groups compared with their corresponding control groups. * $p<0.05$.

apoptosis against Con A-stimulated immune cells and is linked to immune responsiveness to mitogens. In addition, because of this property, emodin could be utilized as a treatment option for cancer and autoimmune diseases caused by abnormally activated immune cells. Autoimmune diseases arise from immune-mediated recognition and destruction of self-tissues (2). Subsequently, principal immune cell types such as T cells, B cells, and DCs multiply to defend against self-components, mistaking them for invasive pathogens (2). Therefore, it is beneficial to find or produce materials like emodin, which exerts an immunosuppressive effect by inducing apoptosis in hyper-proliferative immune cells. Further examination of the specific immune cell suggests that emodin targets would precisely uncover how apoptosis functions to control the delicate equilibrium in a complementary immune system.

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