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The Effects of Emodin on the Expression of Cytokines and Functions of Leukocytes from Sprague-Dawley Rats

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Abstract. *Emodin has been reported to induce apoptosis in many human cancer cell lines, although its effects on leukocyte functions in vitro have not been demonstrated. Therefore, the purpose of this study was to assess the effect of emodin on the phagocytosis of macrophages, the activity of natural killer cells and the expression of cytokines in leukocytes from Sprague-Dawley rats. Leukocytes, isolated from rats, were placed into culture plates for incubation with or without various concentrations of emodin for 1-6 hours and the functions of macrophages and natural killer cells were evaluated by flow cytometric analysis. The results indicated that emodin caused a decrease in phagocytosis of macrophages after treatment for up to 4 hours but 6-hour treatments led to an increase in the phagocytosis of macrophages. Further, emodin increased the activity of natural killer cells, both effects being dose-dependent. The levels of cytokines from the examined leukocytes were evaluated by ELISA and the results indicated that emodin increased the levels of IL-1β and TNF-α, results which were confirmed by PCR assay for the mRNA expressions of the examined cytokines.*

It is well accepted that the cytokines (interleukins 1, 2 and 6 and tumor necrosis factor α (TNF-α)) are involved in a variety of pathological phenomena including lung infection by viruses (1). Dependent on the site which is infected by an antigen, different inflammatory responses are observed (2). Most of the inflammatory cytokines originate from leukocytes.

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Emodin (1,3,8-trihydroxy-6-methylanthraquinone), a natural compound isolated from *Rheum palmatum*, is used in China for the treatment of gallstones, hepatitis, inflammation, osteomyelitis and skin disorders (3, 4). Emodin has been reported to have antibacterial, antifungal and antiviral activities (5-7). Although emodin has been shown to display antiproliferative effects against many tumor cells (8-11), the most potent anticancer activity is against prostate cancer (12). We examined whether or not emodin affects the percentage of viable cells, the levels of cytokines and the functions of leukocytes *in vitro*.

Materials and Methods

Chemicals and reagents. Emodin, LPS, Con A, propidium iodide (PI), ribonuclease-A, Tris-HCl, triton X-100, trypan blue and heparin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Human TNF-α and IL-1 were purchased from Calbiochem-Novachem Corporation (Germany). Dimethyl sulfoxide (DMSO), potassium phosphates and TE buffer were purchased from Merck Co. (Darmstadt, Germany). RPMI 1640 medium, fetal bovine serum (FBS), glutamine, penicillin-streptomycin and trypsin-EDTA were obtained from Gibco BRL (Grand Island, NY, USA).

Animals. Forty-two male Sprague-Dawley (SD) rats, weighing 180-200 g, were obtained from the Animal Center of NSC (Taipei, Taiwan, ROC). The rats were housed in cages and maintained at 25˚C on a 12-h light/dark cycle in the Animal Center of China Medical University (Taichung, Taiwan, ROC), following accepted animal guidelines. The animals had free access to water and chow. All animals were at least 12 weeks of age at the time of sacrifice.

Grouping rats for experiments. Forty-two rats were divided into 6 groups. Group I contained 6 rats for cytotoxicity experiments. Group II contained 6 rats for cytokine experiments. Group III contained 6 rats for natural killer (NK) cell activity experiments. Group IV contained 6 rats for phagocytosis experiments. Group V

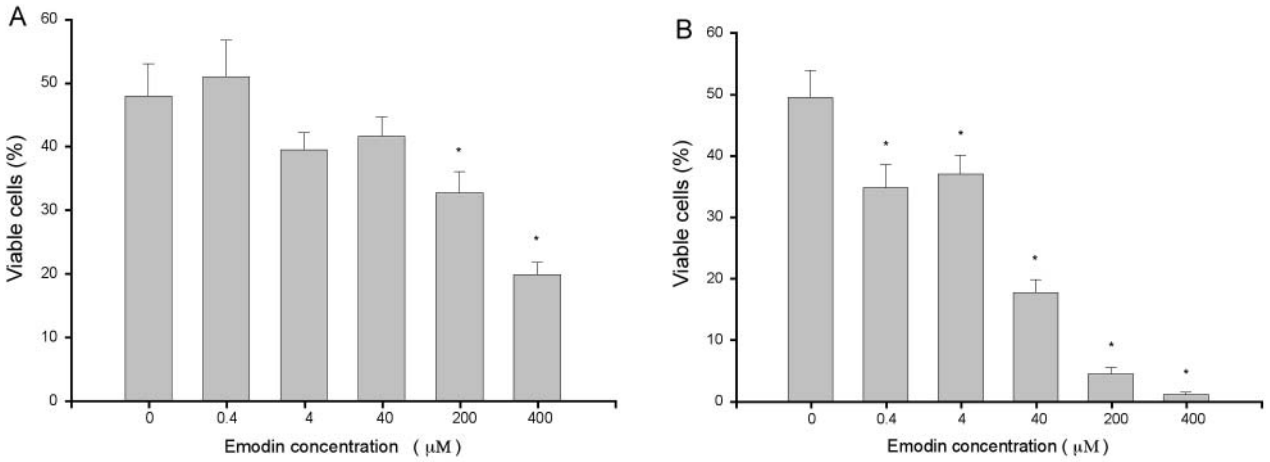

Figure 1. *The percentage of viable leukocytes from rats after emodin treatment. Leukocytes (5x10^5 cells/well; 12-well plates) were cultured in RPMI 1640 medium + 10% FBS with various concentrations of emodin for 12 (panel A) and 24 h (panel B). Then the cells were collected by centrifugation and the viable cells were determined by flow cytometry, as described in Materials and Methods. The data represent mean±S.D. of three experiments. *p<0.05*

contained 6 rats for polymerase chain reaction (PCR) experiments. Group VI contained 12 rats for control experiments.

Isolation of rats' leukocytes. Leukocytes were separated from the whole blood of SD rats by the Ficoll-Paque procedure. Leukocytes, prepared in RPMI 1640 medium with glutamine, were incubated at 37˚C in 95% air and 5% CO_2 for 10 min before cell counting by trypan blue exclusion and flow cytometry (13, 14).

Treatment of leukocytes for cytotoxicity determinations. Approximately 1x10^5 cells in 1 ml of medium with various concentrations of emodin in dimethyl sulfoxide (DMSO) were cultured in each well of a 24-well culture plate, followed by collection of the media. The same volume of vehicle (DMSO) was added to the controls as to the chemically-treated samples. In the cytotoxicity study, the cells were treated with emodin for 12 and 24 h, with no medium change during the treatments. The collected cells were centrifuged at 1000xg for 5 min to remove the media, and counted by trypan blue and flow cytometry for the determination of viable cells (13).

Treatment of leukocytes for determinations of cytokines. The levels of TNF-α, IL-1β and IL-6 were quantified using the following kits (all from R&D Systems, USA): Quantikine Human TNF-α Immunoassay kit, Quantikine Human IL-1β Immunoassay kit, Quantikine Human IL-6 Immunoassay kit, respectively. Assays were performed according to the manufacturer's recommended procedures (13, 14).

Reverse transcription polymerase chain reaction (RT-PCR). The total RNA was extracted from leukocytes which had been treated with or without 4, 40 and 200 μM emodin for 24 h by using the Qiagen RNeasy Mini Kit, as described previously (14, 15). About 1.5 μg RNA, 0.5 μg of oligo-dT primer and DEPC (diethyl pyrocarbonate)- treated water were combined into a 0.5 μL microcentrifuge tube (final volume, 12.5 μL). The entire mixture was heated at 70˚C for 10 min and chilled on ice for at least 1 min. The subsequent procedures for conducting reverse transcription followed those in the instruction manual (First-strand cDNA synthesis kit, Novagen). The sequences of primers are as follows: TNF-α: 3' primer 5'-CAT CTG CTG GTA CCA CCA GTT-3' and 5' primer 5'-TGA GCA CAG AAA GCA TGA TC-3' (396 bp); IL-1β: 3' primer 5'-GGG TTC CAT GGA GAA GTC AAC-3' and 5' primer 5'-CAC CTC TCA AGC AGA GCA CAG-3' (80 bp); IL-6: 3' primer 5'-GAG AGC ATT GGA AGT TGG GG-3' and 5' primer 5'-CTT CCA GCC AGT TGC CTT CT-3' (496 bp); INF-γ: 3' primer 5'-TTACAGATGGTTGTGAGCCACCU-3' and 5' primer 5'-AGACAATCAGCCAAGCCTTGTT-3' (192 bp). Under optimized PCR conditions, all data were collected without saturation or missing bands. Each assay was conducted at least twice to ensure reproducibility (16, 17)

Statistics. The data were expressed as mean±S.D. Logarithmically transformed data were subjected to statistical analyses. Differences between groups were analyzed using the Student's *t*-test and the two-way analysis of variance (ANOVA) with replication. If there was a significant interaction, we judged that the effects of emodin were synergistic. A *p*-value <0.05 was considered to be significant.

Results

Effects of various concentrations of emodin on viability of rats' leukocytes. In the presence of emodin (0.4-400 μM), the cells were collected and stained by propidium iodine and analyzed by trypan blue and flow cytometry. The results indicated that cells were increasingly stained as the time and concentration increased, suggesting that emodin induced cell death of leukocytes. Further increasing the concentration of emodin resulted in a greater decrease of viable leukocytes (Figure 1A and B).

Effects of various concentrations of emodin on the activity of natural killer cells from rats. In the presence of emodin (0.4-400 μM), the NK cells were collected and counted by trypan blue. The results indicated that the target cells were killed by NK cells as the concentration increased, suggesting that emodin induced NK cell activity. These effects were dose-dependent (Figure 2).

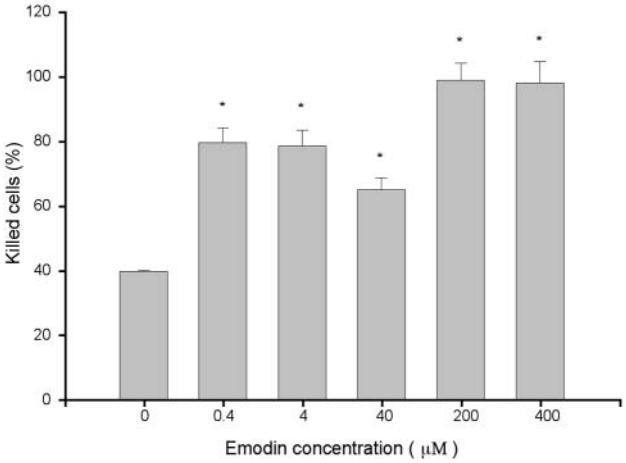

Figure 2. *The percentage of target cells killed by natural killer cells from rats after emodin treatment. Leukocytes (5x10^5 cells/well; 12-well plates) were cultured in RPMI 1640 medium + 10% FBS with various concentrations of emodin for 24 h. Then the cells were collected by centrifugation and the percentage of target cells killed by natural killer cells was determined by flow cytometry, as described in Materials and Methods. The data represents mean±S.D. of three experiments. *p<0.05*

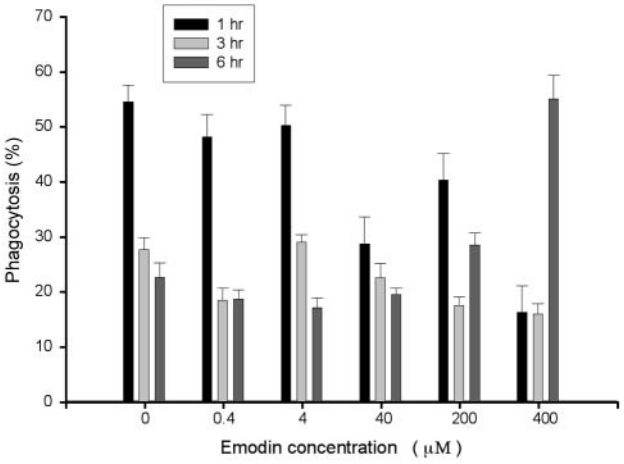

Figure 3. *The percentage of target cells phagocytised by macrophages from rats after emodin treatment. Leukocytes (5x10^5 cells/well; 12-well plates) were cultured in RPMI 1640 medium + 10% FBS with various concentrations of emodin for 24 h. Then the cells were collected by centrifugation and the percentage of target cells phagocytised by macrophages was determined by flow cytometry, as described in Materials and Methods. The data represent mean±S.D. of three experiments. *p<0.05*

Effects of various concentrations of emodin on the activity of macrophages from rats. The results indicated that emodin induced macrophage activity since target cells were phagocytised by macrophages after emodin treatment for 6 h and these effects were dose-dependent as studied by flow cytometric assays (Figure 3). However, 1- to 4-hour treatment with emodin did not affect the macrophage activity (Figure 3).

Effects of various concentrations of emodin on cytokines releases from rats' leukocytes. In the presence of emodin (0.4-400 μM), the leukocytes were collected and counted by trypan blue. No or little IL-1β and TNF-α were detected in the media of untreated cells. Conversely, IL-1β and TNF-α were secreted from the 40 and 400 μM emodin-treated cells. The secretion of IL-1β and TNF-α increased dose-dependently, and their levels in 40 and 200 μM emodin-treated leukocytes were very high (IL-1β: Figure 4A and B; TNF-α: Figure 4C and D).

Effects of emodin on gene mRNA expression in rats' leukocytes. The mRNA gel picture and ratio of cytokine mRNA levels in response to the effect of 40 and 200 μM emodin on leukocytes was examined (Figure 5A and B). Figure 5 shows that IL-1β and TNF-α mRNA levels decreased after 200 μM emodin was added to the cells for 24 -h treatment.

Discussion

Many reports have shown that emodin displays anti-inflammatory action *in vitro*. In this study, we examined the effects of emodin on the cytokines produced by leukocytes as well as leukocyte functions in Sprague-Dawley rats. Emodin induced cytotoxicity in a dose-and time-dependent manner. This is in agreement with other reports (18-20). It has also been found that emodin induced apoptosis in rats' leukocytes based on the appearance of the sub-G1 group from cell cycle analysis (17-19). This was confirmed by our experiments (data not shown).

In the NK cell activity experiments, emodin promoted those cells' killing activity, the percentage of cells being killed increasing with increasing concentrations of emodin in the culture media. In the macrophage activity experiments, emodin promoted the cells phagocytising activity, the percentage of target cells being phagocytised dose-dependently after the increase of emodin in the culture media for 6 h. This finding demonstrated that emodin can protect against antigen infection, which is in agreement with other reports which demonstrated that emodin exhibited hepatoprotective effects on carbon tetrachloride (CCl_4)- as well as D-galactosamine (D-Gala)-induced liver damage (21). The histopathological examination also clearly showed that emodin reduced lymphocyte cells, Kupffer cells, ballooning degeneration, cell necrosis and hyaline degeneration on CCl_4- and D-Gala-induced doseage (21).

To date, emodin has not been reported to induce IL-1β or TNF-α secretions in rats' leukocytes, but the high dose of emodin led to the inhibiton of the examined cytokines mRNA. IL-8 is known to promote liver neutrophil infiltration and activation (22). In the present study, we successfully detected the induction of IL-1β and TNF-α secretions in emodin-treated leukocytes derived from SD rats' blood. IL-1β and TNF-α and

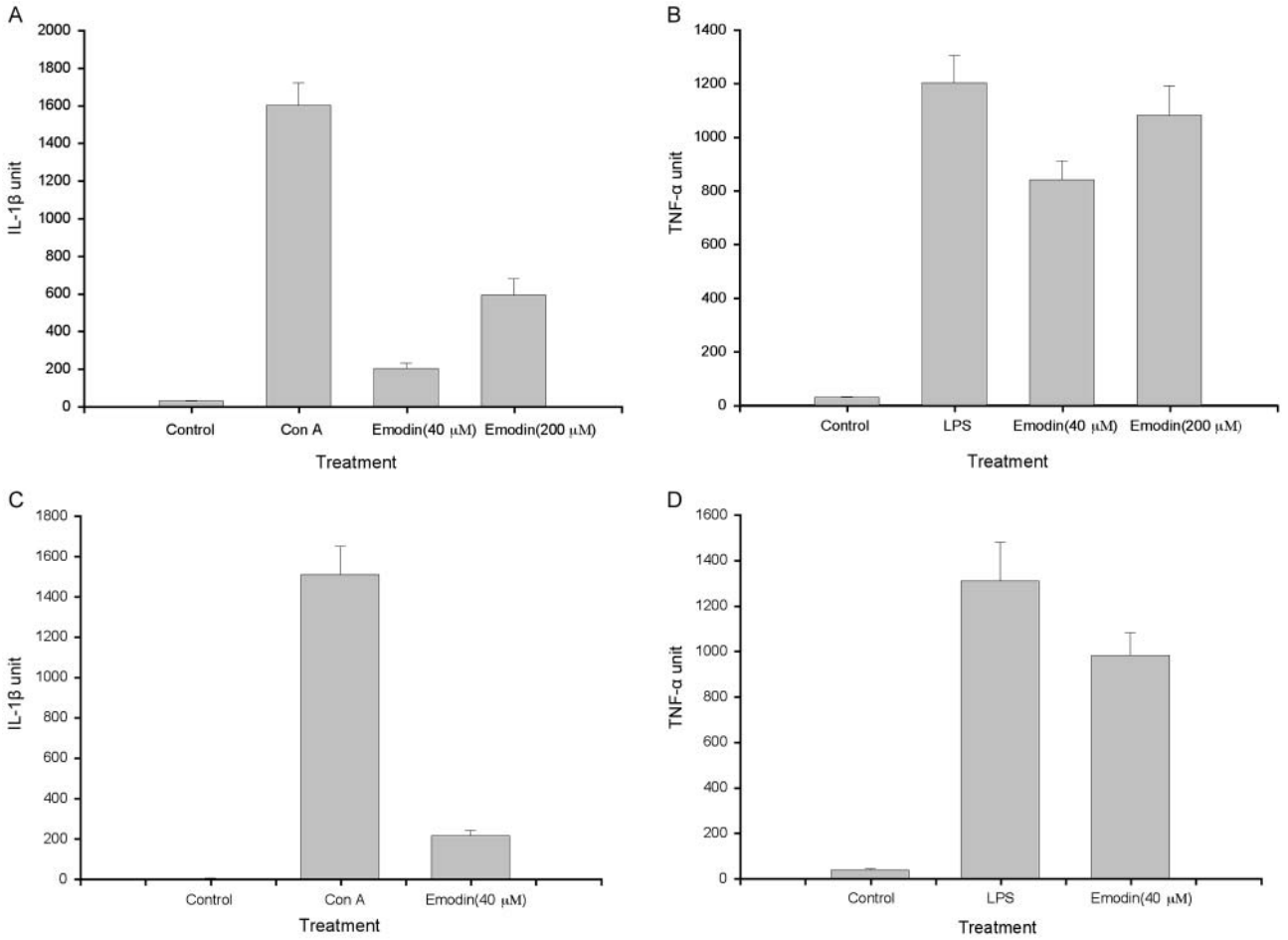

Figure 4. *Effects of emodin on the release of cytokines in rat leukocytes. Leukocytes ($5x10^5$ cells/well; 12-well plates) were cultured in RPMI 1640 medium + 10% FBS with various concentrations of emodin for 24 h. Then the cells were collected by centrifugation and the IL-1β (panel A: Con A pre-treatment; panel B: LPS pre-treatment) and TNF-α (panel C: Con A pre-treatment; panel D: LPS pre-treatment) were analyzed by flow cytometry, as described in Materials and Methods. The data represent mean±S.D. of three experiments. *p<0.05*

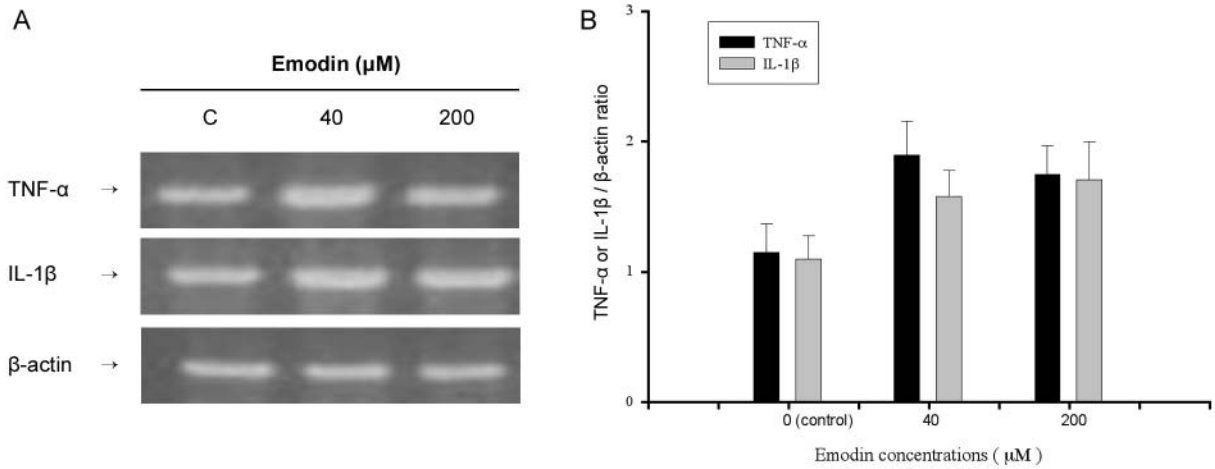

Figure 5. *Effect of emodin on the expression of cytokines mRNA in SD rats' leukocytes. The cells were incubated with 0, 40 or 200 μM emodin for 24 h. The cells were collected to extract RNA. The extracted RNA was subjected to RT-PCR analysis using specific primers for cytokines and β-actin, and then PCR-amplified cDNA derived from mRNA (panel A) were applied to agarose gel-electrophoresis and the ratio of TNF-α or IL-1β (panel B) determined.*

IL-8 were detected and increased in emodin-treated leukocytes, respectively.

It is known that IL-6 is one of the typical basophil-derived cytokines (23). Our results did not show that emodin affects the levels of IL-6. However, to our knowledge, the induction of IL-1β and TNF-α secretions in hepatocyte-derived cells has not been previously reported. In this study, we showed that emodin has the potential to induce IL-1β, IL-8 and TNF-α secretions. Thus, we hypothesize that emodin stimulates macrophages to secrete IL-1β and TNF-α first. IL-8 may recruit neutrophils and monocytes/macrophages to the inflammatory response, respectively. It is well accepted that TNF-α has a pluripotential activity, *e.g.,* activation of neutrophil and macrophage cells and induction of IL-8 secretion, *etc.* (24, 25).

Hence, this is the first report showing that emodin induced the secretion of IL-1β and TNF-α by rat's leukocytes.

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