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Immunomodulatory Activity of Betulinic Acid by Producing Pro-Inflammatory Cytokines and Activation of Macrophages

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Betulinic acid (BA), a pentacyclic triterpene isolated from *Lycopus lucidus*, has been reported to be a selective inducer of apoptosis in various human cancer and shown anti-inflammatory and immunomodulatory properties. We postulated that BA modulates the immunomodulatory properties at least two groups of protein mediators of inflammation, interlukin-1β (IL-1β) and the tumor necrosis factor-α (TNF-α) on the basis of the critical role of the monocytes and tissue macrophages in inflammatory and immune responses. TNF-α and IL-1β were produced by BA in a dose dependent manner at concentration of 0.625 and 10 μg/mL. The production of NO associated with *i*NOS was inhibited when treated with LPS at the concentration of 2.5 to 20 μg/mL of BA whereas COX-2 expression was decreased at 2.5 to 20 μg/mL. These modulations of inflammatory mediators were examined in LPS-stimulated RAW 264.7 cells and peritoneal macrophages. The morphology of macrophage was also examined and enhanced surface CD 40 molecule was expressed when treated BA at 0.625~5 μg/mL with or without LPS. Furthermore, BA (20 μg/mL) enhanced apoptosis by producing DNA ladder in the RAW 264.7 cells. Our results indicated that BA induced activation of macrophage and pro-inflammatory cytokines. This may provide a molecular basis for the ability of BA to mediate macrophage, suppress inflammation, and modulate the immune response.

Key words: *Lycopus lucidus*, Betulinic acid, Macrophage, TNF-α, IL-1β

INTRODUCTION

As a part of an investigation for anti-AIDS agents, betulinic acid (BA) isolated from the leaves of *Syzigium clariflorum* (Myrtaceae) was found to show significant anti-HIV activity (Fujioka *et al.*, 1994). We have studied other species containing BA, the plant natural product, *Lycopus lucidus* (Labiatae) as an immunomodulator, the immunosuppressive properties of ultraviolet (UV) radiation have been known for several years (Krutmann and Elmets, 1988; Roberts *et al.*, 1988). BA (Fig. 1), a pentacyclic triterpene, is an experimental antineoplastic agent that induces apoptosis in melanoma cells *in vitro* and *in vivo* (Pisha *et al.*, 1995), as well as in neuroectodermal tumor cell lines *in vitro* (Fulda *et al.*, 1997). BA is abundant in the plant kingdom and easily prepared in high yields from betulin of white-barked birch trees *(Betula spp.)*. BA and its derivative dihydrobetulinic acid have been identified as anti-HIV agents for their inhibitory activity against HIV-1 replication in acutely infected H9 cells (Hashimoto *et al.*, 1997). Those are also reported to be melanoma specific cytotoxicity agent against cell lines MEL-1-2-3 and 4. These compounds have been shown to completely inhibit

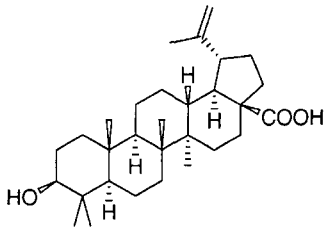

Fig. 1. Chemical structure of betulinic acid

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tumor growth in athymic mice carrying human melanoma (Pisha *et al.*, 1995). The other biological activities reported for BA includes antimalarial (Bringmann *et al.*, 1997), anti-inflammatory (Mukherjee *et al.*, 1997; Recio *et al.*, 1995) and inhibition of phorbol ester induced epidermal ODG in mouse skin model (Yasukawa *et al.*, 1991). The cytotoxicity of BA and its derivatives is probably mediated by the ultimate induction of the apoptotic machinery in melanoma (Pisha *et al.*, 1995), neuroblastoma (Schmidt *et al.*, 1997), medulloblastoma and Ewings sarcoma (Fulda *et al.*, 1997). Different classes of anti cancer drugs may trigger apoptosis by acting on different sub cellular targets and by activating distinct signaling pathways. BA triggers apoptosis by a direct effect on mitochondria (Fulda *et al.*, 1998). These findings have identified BA as a promising anti tumor agent.

TNF is now known to be involved in cellular transformation (Komori *et al.*, 1993), tumor promotion (Suganuma *et al.*, 1999), and induction of metastasis (Orosz *et al.*, 1993; Hafner *et al.*, 1996; Orosz *et al.*, 1995). In agreement with these observations, mice deficient in TNF have been shown to be resistant to skin carcinogenesis (Moore *et al.*, 1999). For several tumors, TNF has been shown to be a growth factor (Giri and Aggarwal, 1998; Aggarwal *et al.*, 1996). Like phorbol ester, TNF mediates the effects in part through activated of a protein kinase C (PKC) pathway (Amott *et al.*, 2002). Similar to TNF, other inflammatory cytokines have also been implicated in tumorigenesis (Hehlgans *et al.*, 2002; Suganuma *et al.*, 2002). Thus, agents that can suppress the expression of TNF and other inflammatory agents have chemopreventive potential (Sueoka *et al.*, 1998; Suganuma *et al.*, 1996).

In order to prove the immunomodulatory function of BA, macrophage cell line RAW 264.7 cells were used as target cells. The BA was isolated from *Lycopus lucidus* leaves portion and stem portion, respectively. Taken together, effects of BA on murine splencyte proliferation and cytokines production were determined.

MATERIALS AND METHODS

Isolation of betulinic acid from *Lycopus lucidus*

The aerial part of *Lycopus lucidus* was collected from may to august in 1992 at Sahmyook University campus (Seoul, Korea). A voucher specimen is deposited at the herbarium of Department of Pharmacy, Sahmyook University, Korea (SYP 92-2). The air-dried plant materials (1.0 kg) were extracted three times for 4 h with hot MeOH under reflux. The MeOH extract was suspended in water, and then fractionated successively with equal volumes of *n*-hexane, chloroform, ethyl acetate, and *n*-butanol. Betulinic acid was obtained from chloroform fraction by silica gel column chromatography and characterized by spectral data (IR, ^{1}H-NMR) and comparison with authentic sample.

Betulinic acid: IR, ν_{max}^{KBR} cm^{-1}: 3420 (OH), 1670 (carboxylic C=O), 1635, 880 (C=C); ^{1}H-NMR : ($CDCl_3$, 300 MHz) δ: 0.77, 0.83, 0.95, 0.97, 0.98 (each 3H, s, CH_3), 1.70 (3H-, s, CH_3-30), 3.01 (1H, m, H-19), 3.19 (1H, dd, J=10.6, 4.5 Hz, H=3), 4.62 (1H, br s, H-29a), 4.74 (1H, br s, H-29b).

Various concentrations of test compounds dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO in the culture media was 0.1%, which did not exhibit any effect on the assay systems (Choi *et al.*, 2000).

Animals

CrjBgi: CD-1 male mice (4 wks) were purchased from Bio Genomics Inc. They were maintained in plastic cages under conventional conditions at laboratory animal center of Sahmyook University.

Preparation of splenocytes

Mice were sacrificed by cervical dislocation under sterile condition. The splenocytes were prepared from the spleens using a method described previously (Cho *et al.*, 1998). Briefly, the splenocytes were released by teasing into a PBS buffer. After removing the red blood cells with an ACE buffer (8 g NH_4Cl, 1 g Na_2EDTA, 0.1 g KH_2PO_4, pH 7.4) single cell suspensions were washed three times in PBS buffer and resuspended in Dulbeccos Modified Eagle Medium (DMEM, Gibco, BRL, Grand Island) with 100 U/mL of penicillin, 100 μg/mL of streptomycin (Gibco, BRL, Grand Isand) and 10% fetal bovine serum (FBS, HyClone, Labs, Logan, UT). The total concentration of cells was 1×10^6 cells/mL.

Proliferation of splenocytes

The splenocytes were treated with LPS (lipopolysaccharide, 10 μg/mL), Con A (Concanavalin A, 4 μg/mL), LPS/BA (1.25, 2.5, 5, 10 μg/mL), Con A/BA (1.25, 2.5, 5, 10 μg/mL) all which were treated with DMEM-10. They were instilled into each well 2×10^5 cells/200 μL/96 well and were cultured in a CO_2 incubator (37°C, 5.5% CO_2 incubation) for 3 days. A cell Titer 96 Aqueous Non-Radioactive cell proliferation Assay Kit (XTT: Promega. WI, USA) were used to measure the proliferation on the splenocyte. Subsequently, 50 μL XTT (sodium 3'-[1-(phenylaminocarbonyl)-3,4-terazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate) reagent added to each well, which were then cultured under the same conditions for 3 h and measured by an ELISA reader (Molecular Devices, U.S.A.) at 490 nm.

Nitric oxide (NO) production assay

Flat-bottomed 96 well, LPS (10 ng/mL), LPS/ BA (0.625, 1.25, 2.5, 5 μg/mL), media only (DMEM-10), RAW 264.7 cell line and Griess reagent (mixture of equal volume of

0.2% *N*-(1-naphthyl) ethylenediamine dihydrochloride in H_2O and 2% sulfanilamide in 5% H_2PO_4) were used as the materials in this study. NO production was carried out according to the method reported by Stuehr and Nathan (1989). The following: LPS (10 ng/mL), untreated mitogen, were prepared as the treated groups. 4 wells per group were used and 200 μL of the cells (1×10^6 cells/mL) was added to each well. The plates were incubated overnight and 100 μL from the surface of each well was transferred into new plate. The new plate was then incubated for 10 minutes with 100 μL of Griess reagent at room temperature and was measured by an ELISA reader at 540 nm.

NO production of peritoneal macrophages

Murine macrophages were prepared from peritoneal exudates cells of mice injected intraperitoneally 2 days previously with 2.0 mL 3% thioglycollate broth. Single cell suspensions were washed three times in PBS buffer and resuspended in DMEM-10. Macrophages were purified by adherence to plastic for 2 h (37°C, 5.5% CO_2 incubation).

The macrophages obtained after 2 h adherence period in DMEM 1640 media were scraped. Peritoneal macrophages (2×10^6 cells/mL) from mice were treated with BA (1.25, 2.5, 5, 10, 20 μg/mL) in the presence of absence of LPS (25 ng/mL) for 24 h. The plates were incubated for 24 h and 100 μL from the surface of each well was transferred into new plate. The new plate was then incubated for 10 minutes at room temperature and was measured by an ELISA reader at 540 nm.

Cytokines bioassays

The RAW 264.7 cells were cultured in DMEM with 10% FBS in 6 well plates at a density 1×10^6 cells/well. Cells were treated in the presence or absence of BA at different concentration (0.625, 1.25, 2.5, 5 μg/mL) with LPS 100 ng/mL, BA (0.625, 1.25, 2.5, 5 μg/mL) for 48 h at 37°C and 5.5% CO_2 incubator. And then each supernatant was collected for cytokines bioassays.

IL-1β assay: In order to measure the IL-1β activity, the ability of the stimulated monocyte supernatants to cause proliferation of the IL-1β dependent T-cell line was assessed. An example was the conalbumin-specific D10 G4.1 murine T-cell clone (ATCC), which requires IL-1β (1 U/mL) and those without in different culture supernatants of BA (5, 10, 15, 20, 25 μL) were prepared as the treated groups. 4 wells per group were used and 100 μL of the cell suspension (4×10^4 cells/well) were added the each well and incubated in humidified air with 5.5% CO_2 for 48 h 37°C. Subsequently, 50 μL of the XTT reagent was added to each well, cultured under the same conditions for 3 h. Then they were measured in an ELISA reader at 490 nm.

TNF-α assay: In order to investigate the TNF activity, the ability of the stimulated monocyte supernatants to lyses a highly TNF-α sensitive murine fibrosarcoma cell line, WEHI-164-JD (ATCC), was assessed (Djeu *et al.*, 1988). The TNF-α production assay was carried out according to the Manual of Clinical Laboratory Immunology (Noel *et al.*, 1991). The WEHI-164 cells were prepared in different culture supernatants of the BA (5, 10, 15, 20, 25 μL) treated groups. 4 wells per group were used and 100 μL of the cell suspension (2×10^4 cells/well) were added the each well. The plates were then incubated in humidified air with 5.5% CO_2 for 48 h 37°C. Subsequently, 50 μL of the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) reagent was added to each well, cultured under the same conditions for 3 h. Then they were measured in an ELISA reader at 540 nm.

Western blot analysis

Western blotting was used to characterize COX-2 of LPS-treated RAW 264.7 and LPS without. The cells were washed with PBS saline and homogenized on ice with a lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.02% NaN_3, 100 μg/mL PMSF, 1 μg/mL aprotinin, 1% triton X-100). The homogenates were centrifuged at 15,000 rpm for 30 min. Whole-cell protein extracts were resuspended in 20 μL of gel sample buffer (10% glycerol, 5% 2-mercaptoethanol, 4% SDS, 62.5 mM Tris-HCl, pH 6.75, 0.01% bromophenol blue). After incubation in a boiling water bath for 5 min, samples were stored at -20 °C. Protein (68 μg) from cell lysate samples were resolved on 12% polyacrylamide gels by SDS-PAGE, followed by electrophoretic transfer to nitrocellulose membrane (NC; Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, England). NC membranes were blocked for 1 h at room temperature in 5% skim milk in PBST buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, 0.05% Tween 20). It was then incubated with anti-COX-2 monoclonal antibodies as the primary antibodies. The antibodies were used at 1:200 dilutions at room temperature for 2 h. After washing 3 times with PBST, blots were then incubated with 1:1000 of horseradish peroxidase-linked anti-mouse IgG (Santa Cruz Biotechnology, Inc., California, USA) for 2 h. The specific protein was detected by a Western blot kit substrate for phosphatase (LumiGLO System, KPL, USA).

Cell staining

To determine the effects of BA on the macrophage morphology, the cells were cultured in sterile glass-slide chambers at a density of 1000 cells/well for 48 h. The culture medium was removed and the cells were treated with either LPS (10 ng/mL), LPS/BA (0.625, 1.25, 2.5, 5 μg/mL) or BA (0.625, 1.25, 2.5, 5 μg/mL) only for 2 days. Following the treatment, the culture supernatant was removed. Cells were fixed and stained in Diff-Quick solution.

Flow cytometry

The RAW 264.7 cells (3×10^6 cells/10 mL) were cultured for 2 days. The cells were then incubated in the presence of either LPS (100 ng/mL), LPS/BA (1.25, 2.5, 5 μg/mL)or media only, BA (1.25, 2.5, 5 μg/mL). The treated cells were then scraped into 0.1% sodium azide with 1% bovine serum albumin and washed twice in a washing buffer at 4°C. Before staining the cells with mono-clonal antibodies, the cell surface Fc receptors were blocked by incubating the cells with purified anti-CD16 for 30 min at 4°C. The residual antibodies were removed by washing. Anti-I-A^b-FITC (Fluorescein isothiocyanate), Anti-CD80-PE (Phycoerythrin), Anti-CD86-FITC, Anti-CD40-PE were then added and cells were stored at 4°C for 30 min. The cells stained with mouse IgG-FITC served as the control for nonspecific binding. The cells were then washed and fixed in cold PBS containing 1% paraformaldehyde (pH 7.2). Flow cytometry analysis was performed using an EPICS V analyzer (Coulter, Hialeah, EL). The fluorescence intensity was determined on the 10,000 cells from each sample using logarithmic amplification.

DNA isolation and electrophoresis

RAW 264.7 cells (1×10^6 cells/10 mL) were pre-incubated at 37°C for 24 h. After cultivation, the cells were treated with BA (5, 10, 20 μg/mL) for 24 h and the medium was removed and pellets were washed three times with a PBS solution. DNA was isolated without delay as described below. After added 500 μL of lysis buffer (0.1 M Tris-HCl, 0.5% Triton X-100, 100 μg/mL protease K, pH 7.8) to cells and were incubated for 3 h at 55°C in a water bath. Then, 1 mL of a phenol:chloroform:isoamylalcohol mixture (25:24:1) were added and samples were mixed by inversion.

The aqueous phase containing the DNA was separated from the organic phase by centrifugation at 15,000 rpm for 30 min at 4°C with the gel plug forming a barrier between these phases. The aqueous phase remained on top of the gel plug. This step was repeated once adding 1 mL of the phenol:chloroform:isoamylalcohol mixture to the same tube and centrifuging the samples for 30 min at 15,000 rpm. After the final centrifugation, the top aqueous layer was poured into a clean polypropylene tube and DNA was precipitated with an equal volume of ethanol. After centrifugation, it was air-dried and resuspended in 30 μL TE buffer (100 mM Tris-Cl, pH 8.0, 1 mM Na_2EDTA). 1 U RNase was added for removing RNA and was incubated for 20 min at 37°C. Purity of DNA was determined by measuring the absorbance at 260 nm (A_{260}) and 280 nm (A_{280}), respectively and using the A_{260}/A_{280} ratio as an indicator of purity. Plastic tubes stored at -20°C for preventing discoloration. One of genomic DNA was run with loading dye (Promega, WI, USA), size-fractionated on 2% agarose gel containing 0.5 μg/mL ethidium bromide. DNA fragment was measured by gel documentation system.

Statistical analysis

Nitrite and cytokines productions were expressed as means±SD of two to six similar independent experiments. Statistical significance was determined using the Student-*t*-test.

RESULTS

Effects on splenocytes proliferation

In order to investigate the effect of BA on lymphocyte proliferation, the splenocytes were incubated with either LPS, mitogen from B-lymphocytes, Con A, or mitogen from T lymphocytes, in the presence of various BA concentrations. In this assay, LPS was added to the splenocytes at a concentration of 10 μg/mL. BA alone induced minimal lymphocyte proliferation (data not shown), Con A or LPS did not stimulate lymphocyte proliferation, and much less proliferation was observed by the combined action of BA and Con A or LPS. The proliferation of the T lymphocytes treated by Con A combination with BA was significantly lower at 10 μg of BA, than when treated with 4 μg/mL Con A only. However, the number of total lymphocytes decreased with increasing BA concentrations (1.25 to 10 μg/mL), as shown in Fig. 2. The phase of the Con A stimulated lymphocytes also followed the same trend (Fig. 3). When the cells were treated with high concentrations of BA (5 to 10 μg/mL) cytotoxicity in the splenocytes was observed.

Effect of BA on NO production

RAW 264.7 cells, a murine macrophage cell line, were used to assess the effect of BA on NO synthesis in a

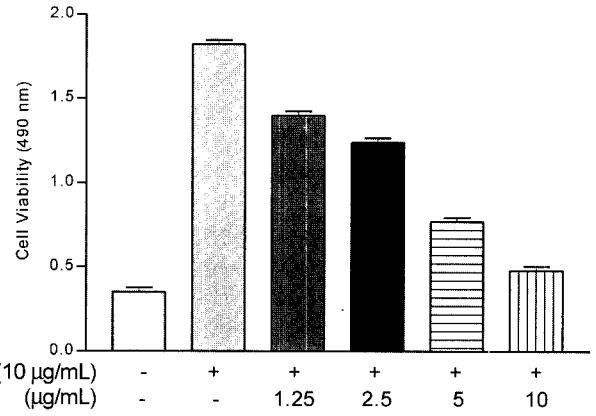

Fig. 2. Proliferous effect of Betulinic acid (BA) by LPS (10 μg/mL)-stimulated murine splenocytes. The cells were purified from spleen of mouse. And treatment of BA with LPS was used at a concentration of 10 μg/mL different amount of BA (1.25, 2.5, 5, 10 μg/mL). After 72 h culture, the cultures pulsed for 18 h with 1 mg/mL XTT in combination with PMS for proliferation XTT assay.

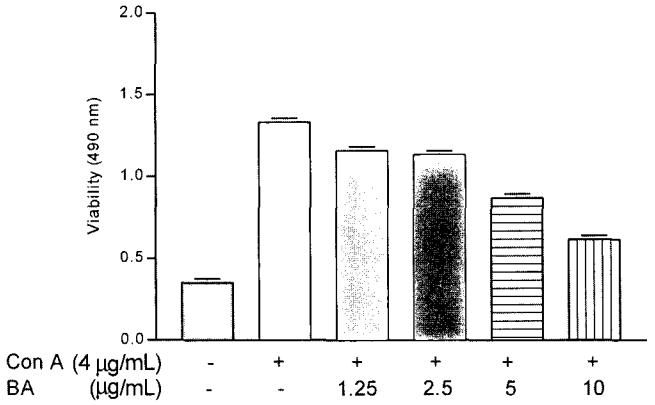

Fig. 3. Proliferous effect of Betulinic acid (BA) by Con A (4 μg/mL)-stimulated murine splenocytes. Cells were purified from spleen of mouse. And treatment of BA with con A was used at a concentration of 4 μg/mL different amount of BA (1.25, 2.5, 5, 10 μg/mL). After 72 h culture, the cultures pulsed for 18 h with 1 mg/mL XTT in combination with PMS for proliferation XTT assay.

model macrophage-mediated inflammatory events *in vitro*. LPS was used as a positive control for macrophage activation. The cells were stimulated with LPS (10 ng/mL) for 18 h, the levels of nitrite, stable oxidized product of NO, increased in the culture medium. The cells treated with BA didn't appear on NO production. When combination of LPS with BA inhibited NO production in a dose-dependent manner and BA (2.5, 5, 10, 20 μg/mL) was decreased by 12.5, 27.3, 51.5, 55.5% when compared to the LPS control (Fig. 4). But this is because one type of BA character appeared to increase the cytotoxicity in the RAW 264.7 cells at certain BA concentration (10 and 20 μg/mL) (data not shown). These results suggest that low BA concentration (0.625~2.5 μg/mL) may function, at least in part, through the macrophage activation in the host defense response. We further examined the effects of BA on NO production in peritoneal macrophages. As seen with RAW 264.7 macrophages (Fig. 4), BA also inhibited NO production in peritoneal macrophages when stimulated with LPS (25 ng/mL) (Fig. 5). And 5~20 μg/mL concentrations of BA inhibited by 12.7, 24.7 and 46.3% compared to the positive control. Cytotoxic effect of BA was not observed under the same experimental condition, as measured by tryphan blue staining (data not shown).

Cytokine production in response to BA

To determine whether BA (0.625, 1.25, 2.5, 5, 10 μg/mL) had a direct effect on cytokines production, a biological assessment of TNF-α activation was measured by using 10 μL of culture supernatant of macrophage cell line RAW 264.7. The proliferation assay was done using TNF-α sensitive cell line WEHI 164, which die with the treatment of culture soup containing TNF-α. As shown in Fig. 6, TNF-α production was increased from 0.625 to 2.5 μg/mL but decreased from 5 to 10 μg/mL. When macrophages were stimulated with LPS (10 ng/mL) decreased in a dose-dependent manner (Fig. 7). We determined TNF-α production antagonized in a dose dependent manner by RAW 264.7 cells stimulated with LPS. IL-1β activation was measured by using 20μL of supernatant in macrophage cell line. IL-1β production had a tendency to like TNF-α production in RAW 264.7 cell without LPS. The proliferation assay was done using IL-1β dependent T cell line (Fig. 8).

Effect of BA on macrophage morphology

Normal RAW 264.7 cell, when cultured in medium alone, tended to be round. Fig. 9 showed that the cells

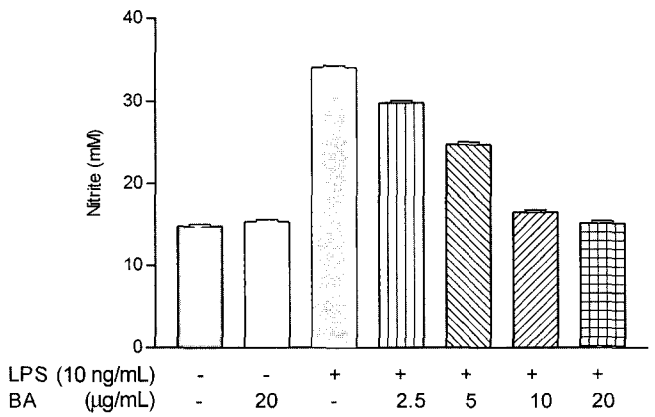

Fig. 4. Inhibitory effects of betulinic acid (BA) on the nitric oxide production in the LPS-stimulated murine macrophages RAW 264.7 cells. Cultures were incubated with LPS (10 ng/mL) in the presence of different amount (2.5, 5, 10, 20 μg/mL) of BA. After cultured for overnight with LPS or without, 100 μL the supernatants were reacted for 10 min at room temperature with Griess reagent.

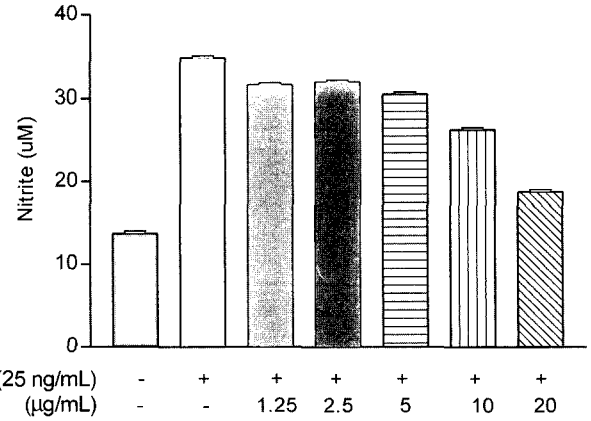

Fig. 5. Effects of betulinic acid (BA) on production of nitric oxide in the peritoneal macrophages. Peritoneal macrophages (2×10^6 cells/mL) from mice were treated with BA (1.25, 2.5, 5, 10, 20 μg/mL) in the presence of absence of LPS (25 ng/mL) for 24 h. After cultivation, 100 μL the supernatants were reacted for 10 min at room temperature with Griess reagent.

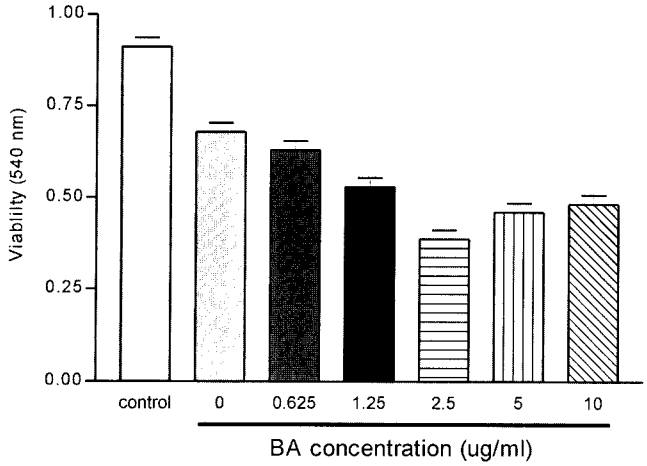

Fig. 6. TNF-α production by betulinic acid (BA) in murine macrophage cell line. RAW 264.7 (2×10^5 cells/mL) were cultured with BA (0.625, 1.25, 2.5, 5, 10 μg/mL) for 48 h. Bioassay of TNF-α was measured by 10 μL of supernatants in the RAW 264.7 cells was added to TNF-α sensitive cell line, WEHI-164.

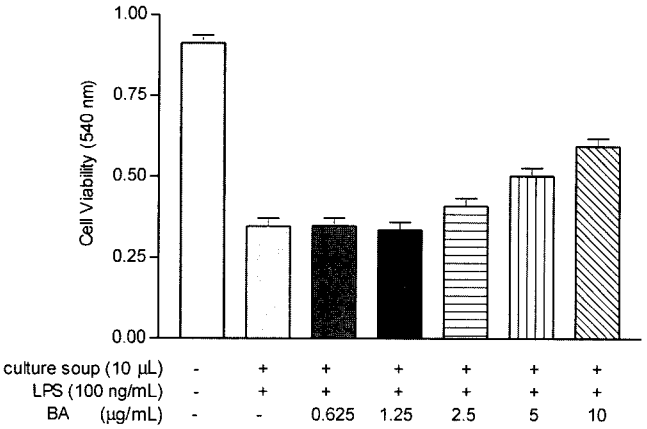

Fig. 7. LPS-induced TNF-α production by betulinic acid (BA) in murine macrophage cell line. The RAW 264.7 cells (2×10^5 cells/mL) were cultured with BA (0.625, 1.25, 2.5, 5, 10 μg/mL) for 48 hrs in the presence of LPS (10 ng/mL). Bioassay of TNF-α was measured by 10 μL of supernatants in the RAW 264.7 cells was added to TNF-α sensitive cell line, WEHI-164.

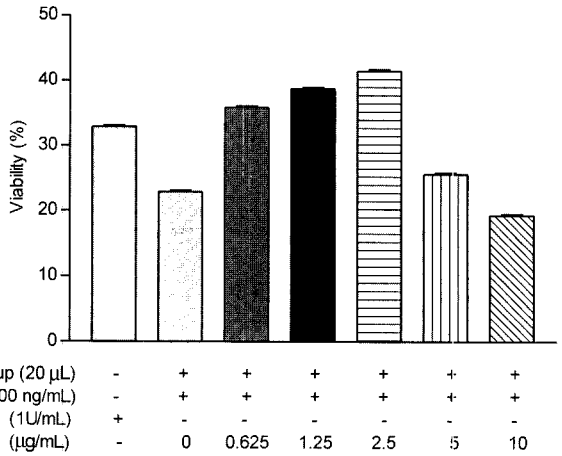

Fig. 8. IL-1β production by betulinic acid (BA) in LPS-stimulated murine macrophage cell line. The RAW 264.7 cells (4×10^5 cells/mL) were cultured with the indicated. Doses of BA (0.625, 1.25, 2.5, 5, 10 μg/mL) for 48 h. Bioassay of IL-1β was measured in IL-1β dependent cell lines, D10S. 20 μL of supernatants in the RAW 264.7 cells was added to IL-1β dependent cell line, D10S.

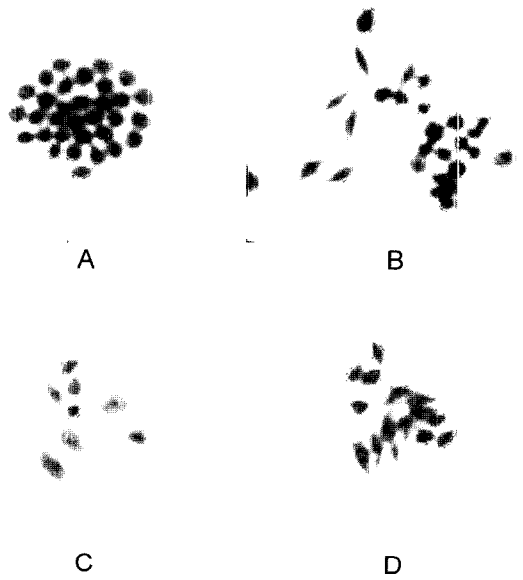

Fig. 9. Macrophage morphological changes in response to betulinic acid (BA). The RAW 264.7 cells were cultured on cover slips in the presence of different concentration of the medium (A), BA (1.25 μg/mL)/LPS(B), BA (2.5 μg/mL)/LPS (C) , BA (5 μg/mL)/LPS (D) and LPS (10 ng/mL: data not shown) for 48 h. The cells were fixed and stained in Diff-quick solution.

treated with BA (2.5 μg/mL)/LPS (Fig. 9C) were larger and rougher than those exposed to either BA (1.25 μg/mL)/LPS (Fig. 9B) or BA (5 μg/mL)/LPS (Fig. 9D).

COX-2 protein expression

Western blot analysis of cells lysates prepared from macrophage cell line conformed constitutive expression of COX-2 protein in RAW 264.7. COX-2 expression in LPS-stimulated 264.7 cells was significant decreased in a dose dependent manner, with maximum inhibition (BA 2.5 μg/mL)/LPS (Fig. 10). Two peptide bands of 72 and 74 kDa co-migrated with the COX-2 protein positive control and were identified as the COX-2 protein doublet. It is likely that these peptides represent different *N*-glycosylation states for the enzymatically active COX-2 molecules as described by Otto *et al.* (1993).

Effects of BA on surface molecule expression

The RAW 264.7 cell surface expression of I-A^b, CD80,

LPS (100 ng/mL)	+	+	+	+
BA (μg/mL)	0	0.6	1.2	2.5

Fig. 10. Western blot analysis of the COX-2 in lysates of RAW 264.7 cells (68 μg protein/lane). The treated cells incubated with LPS (100 ng/mL) and in combination with either betulinic acid (0.625, 1.25, 2.5, 5 μg/mL) were separated by 12% SDS PAGE, transferred to NC membrane and incubated with a mouse monoclonal IgG_1 to COX-2 (1:200 dilution). After incubation with goat anti-mouse secondary antibody (1:1000 dilution), the COX-2 bends were detected using substrate for phosphatase.

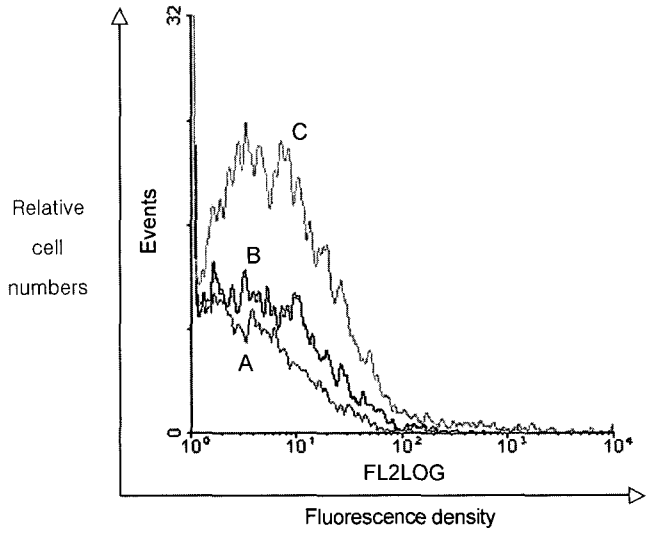

Fig. 11. Expression of the co-stimulatory molecules CD 40 expression. The RAW 264.7 cells cultured in the presence of medium(A), BA 2.5 μg/mL (B), LPS/BA 2.5 μg/mL (C) for 48 h. The surface CD 40 molecule was labeled with anti-CD 40-PE and the cells were stained using anti-Vβ2-PE, which served as an isotype control for the nonspecific binding (data not shown). The X-axis shows the fluorescence density. The Y-axis shows the relative cell numbers. The shaded curve denotes the background fluorescence.

CD86 and CD40 were examined by flow cytometry (Fig. 11). The major B-cell co-stimulatory signal is CD40, which are closely related members of the TNF receptor family. It is involved in directing all phases of the B-cell response. Binding of CD40 by CD40 L helps to drive the resting B cell into the cell cycle and is essential for B-cell responses to thymus-dependent antigens. The surface CD40 molecule was expressed on resting cells (Fig. 11B) treated with BA (2.5 μg/mL). However, the surface $I\text{-}A^b$, CD80 and CD86 molecules were not expressed on the resting cells. Also The $I\text{-}A^b$, CD80 and CD86 were not expressed after exposure to BA (not data shown). However, expression of CD40 was observed on the cells treated with BA (2.5 μg/mL)/LPS (100 ng/mL) (Fig. 11C).

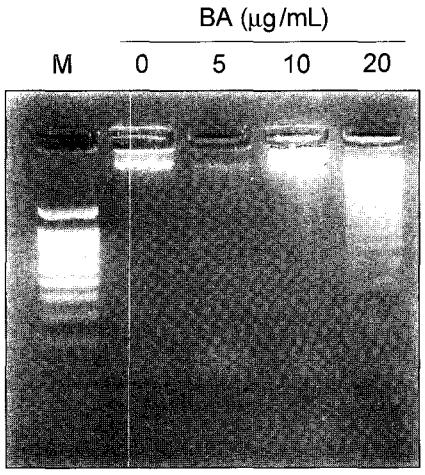

Fig. 12. Betulinic acid (BA) induced apoptosis in the RAW 264.7 cells. RAW 264.7 (1×10^6 cells/10 mL) cells were incubated with BA (0, 5, 10, 20 μg/mL) for 24 h. At the times indicated, the cells were lysed and DNA was prepared. DNA (1 μg/lane) fragmentation was analyzed by 2% agarose gel electrophoresis.

BA induced apoptosis

To demonstrate size and quality of DNA isolated after treating different concentration of BA (5, 10, 20 μg/mL). We performed agarose gel electrophoresis for measuring apoptosis. Apoptotic cells that have lost their membrane integrity appear orange due to co-staining with ethidium bromide. DNA (1 μg) from treatment BA (20 μg/mL) was shown apoptosis by DNA ladder (Fig. 12).

DISCUSSION

The present study was undertaken to elucidate the modulatory effects of BA on the production of inflammatory mediators in RAW 264.7 cell. We showed that BA increases the production of TNF-α, IL-1β in the cell and expresses CD40 molecule in LPS stimulated cell or not.

This suppression was correlated with up-regulation of TNF-α, IL-1β and CD40. BA suppresses the production of NO and COX-2 in LPS-stimulated RAW 264.7 cells. LPS (25 ng/mL) stimulated peritoneal macrophages are decreased NO production by BA in a dose dependent manner.

Nitric oxide and prostaglandins, which are produced by *i*NOS and COX-2, respectively, have been implicated as important mediators in end toxemia and inflammatory conditions (Ahmad *et al.*, 2002). It has been demonstrated that NO plays a pivotal role as neurotransmitter, vasodilator and immune regulator in a variety of tissues at physiological concentration (Moncada *et al.*, 1991). High levels of NO produced by *i*NOS, however, have been defined as a cytotoxic molecule in inflammation and endotoximia (Krnocke *et al.*, 1997). PGE_2, like NO is a pleiotropic mediator produced at inflammatory sites by

COX-2 and gives rise to pain, swelling and stiffness (Seibert *et al.*, 1994). Thus, potential inhibitors of *i*NOS and COX-2 have been considered to be anti-inflammatory drugs. We here demonstrated that BA inhibits significantly the gene expression of COX-2 in LPS-stimulated RAW 264.7 cells.

Most effector T cells express members of the TNF protein family as cell surface molecules. The most important TNF family proteins in the T-cell effector function are TNF-α and TNF-β (which can also be produced as secreted molecules), the Fas ligand and the CD40 ligand, the latter two always being associated with the cell surface.

TNF-α was produced by Th_1 cells, some Th_2 cells and cytotoxic T cells in both a soluble and membrane-associated form and can also deliver activating signals to macrophages. Some members of the family of TNF receptors can stimulate apoptosis (Charles *et al.*, 1999). Th_2 cells express B cell activating effectors molecules, whereas Th_1 cells express effector molecules that activate macrophages. TNF-α production was increased by dose dependent manner of BA (from 0.625 to 2.5 μg/mL). Expression of surface molecule CD40 was also increased when treated with BA in the presence or absence of LPS. Normal RAW 264.7 cells, when cultured in medium alone, tended to be round. None appeared to spread over the surface (Fig. 9A). Fig. 9 shows that the cells treated with BA (1.5, 2.5 μg/mL)/LPS (Fig. 9B/9C) were larger and rougher than those exposed to medium only (Fig. 9A) or BA (5 μg/mL)/LPS (Fig. 9D).

Our results demonstrated that BA inhibits the production of NO in LPS-stimulated macrophage. This effect occurs by down-regulation of COX-2 protein expression. BA also produces TNF-α and TNF-α helps expression of CD40 for up-regulation. BA was found stimulate macrophages to release TNF-α , IL-1β and Inhibition of NO production, cell morphological changes and surface molecule expression were largely affected by BA. However, these effects were potentiated by LPS addition. Therefore, these results demonstrate the ability of BA to activate RAW 264.7 cells both directly for cytokine production and indirectly for NO inhibition and stimulate the expression of some of the surface molecules.

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