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The Effect of Linarin on LPS-Induced Cytokine Production and Nitric Oxide Inhibition in Murine Macrophages Cell Line RAW264.7

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The herb, *Chrysanthemum zawadskii* var, *latilobum* commomly known as Gu-Jul-Cho in Korea, used in traditional medicine to treat pneumonia, bronchitis, cough, common cold, pharyngitis, bladder-related disorders, gastroenteric disorders, and hypertension. Linarin is the main active compound and the biological mechanisms of its activity are unclear. It is believed that effects of this herb may be exerted through the pluripotent effectors of linarin due to its ability to treat a variety of afflictions. In this study, the effects of linarin on the mouse macrophages cell line, RAW 264.7, were investigated. It was found that linarin could activate macrophages by producing cytokines. Monocytes and tissue macrophages produce at least two groups of protein mediators of inflammation, interleukin 1 (IL-1) and the tumor necrosis factor (TNF). Recent studies have shown that TNF and IL-1 modulate the inflammatory function of endothelial cells, leukocytes, and fibroblasts. TNF-α production by macrophages treated with linarin occured in a dose dependent manner. However, IL-1 production was largely unaffected by this natural product. This study demonstrated the ability of linarin to activate macrophages both directly and indirectly. Linarin also affect both cytokine production and nitric oxide inhibition, in addition to the expression of some surface molecules. Nitric oxide (NO), derived from L-arginine, is produced by two forms(constitutive and inducible) of nitric oxide synthase (NOS). The NO produced in large amounts by inducible NOS is known to be responsible for the vasodilation and hypotension observed in septic shock. Linarin was found to inhibit NO production in the LPS-activated RAW 264.7 cells. Linarin may be a useful candidate as a new drug for treating endotoxemia and the inflammation accompanied by NO overproduction. The linarin-treated total lymphocytes exhibited cytotoxicity in a dose dependent manner between 20 μg/ml and 40 μg/ml. These results suggest that linarin may function through macrophage activation.

Keywords: *Chrysanthemum zawadskii* var, *latilobum*, Macrophage activation, Inflammation, TNF-α, IL-1, iNOS

INTRODUCTION

The extract from *Chrysanthemum zawadskii* var, *latilobum*, commonly known in Korea as Gu-Jul-Cho, is used in traditional medicine to treat pneumonia, bronchitis, cough, common cold, pharyngitis, bladder-related disorders, women's diseases, gastroenteric disorders, and hypertension (Lee,1982). Linarin is the main compound of *Chrysanthemum zawadskii* var, *latilobum* (Fig. 1). It is an acacetin-7-β-rhamnoglucoside, called aca-ciin. Linarin has significant beneficial therapeutic effects. However, the biological mechanisms of its activity are unclear. Because of this wide diversity in biological activity, a common mechanism involving macrophage activation has been suggested (Im, 1998). For this reason, the effects of linarin on macrophage function were examined.

Macrophage activation is normally mediated by a wide variety of mechanisms (Fig. 2). These include exposure to cytokines such as interferon-γ (IFN-γ) and interleukin-2 (IL-2). They are also activated by exposure to bacteria and bacterial products (Adams and Hamilton, 1984). Macrophages can destroy tumor cells after being treated with both recombinant IFN-γ and bacterial LPS

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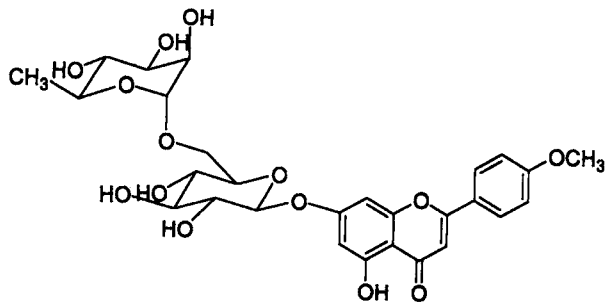

Fig. 1. Structure of Linarin(acacetin-7-rhamnoglucoside)

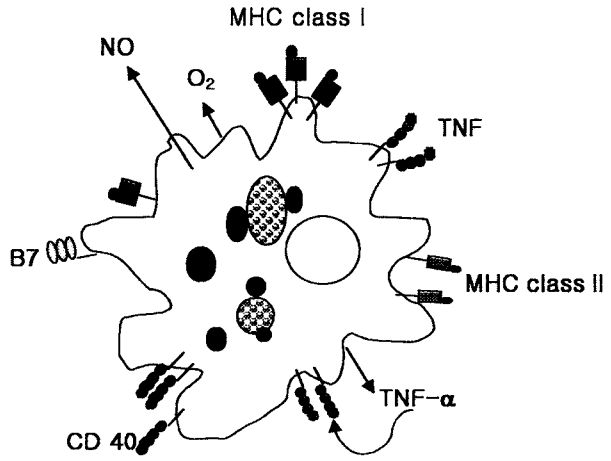

Fig.2. Activated macrophages undergo changes that greatly increase their antimicrobial effectiveness and amplify the immune response. Activated macrophages increase the expression of the CD40 and TNF receptors, and secrete TNF-α. This autocrine stimulus synergizes with the IFN-γ secreted by T_H1 cells to increase the antimicrobial action of macrophages, particularly by inducing the production of nitric oxide (NO) and oxygen radicals (O_2). The macrophages also upregulated their B7 proteins in response to CD40 ligand binging on the T cell, and increases the expression of class II MHC molecules, thus allowing further activation of resting CD4 T cells.

suggesting that at least two stimuli are required for complete activation (Drysdale *et al.*, 1988; Moore *et al.*, 1991). It has been shown that each of these molecules interact with specific high affinity receptors located on the macrophage plasma membrane (Lor-sbach *et al.*, 1993).

Mononuclear phagocytes have also emerged as cells central to the inflammation sites (Morrison *et al.*, 1979). Although these cells release a plethora of inflammatory mediators, two of the more important cytokines, interleukin (IL-1) and the tumor necrosis factor (TNF), appear to play an important role in the immune process. Both IL-1 and TNF have a profound effect on tissue remodeling, repair, and inflammation by coordinating the activities of many other cells, including endothelial cells, granulocytes, osteoclasts, fibroblasts, hematopoietic cells, and lymphoid cells (Djeu *et al.*, 1988). An important concept emerging from these studies is that under certain physiological conditions, cytokines play a role in the homeostasis of cells and tissue (Pohlman *et al.*, 1986). Inflammation processes and the response to injury are associated with the augmented release of these protein mediators and toxic or life-threatening syndromes, e. g., cachexia, shock, ect, result from the exaggerated and prolonged release (Linna and Ian, 1996).

Nitric oxide (NO) is a multi-functional mediator, which is implicated in a wide variety of biological functions such as neurotransmission, non-specific immune defense and vasodilatation (Moncada *et al.*, 1991). IL-1 and TNF both play an important role during the various phases of disease, since they have a direct impact on an array of cellular targets (James and Steven, 1988).

The aim of this paper was to examine the effects of linarin on macrophage functions, NO production, cytokine release, and expression of some adhesion molecules.

MATERIALS AND METHODS

Linarin

Linarin was isolated from Chrysanthemum zawadskii var, latilobum by methanol extraction and was confirmed as a single pure compound by instrumental analysis from the Pharmacognosy Laboratory in Sahmyook University. Various concentrations of the test compounds were dissolved in dimethylsulfoxide (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

Eight-week old female BALB/cmice were purchased from Daehan Biolink. They were maintained in plastic cages under conventional conditions at the laboratory animal center in Sahmyook University.

Splenocyte preparation

The mice were sacrificed by a cervical dislocation under sterile conditions. 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 RPMI1640 medium supplemented with 20 μM HEPES buffer. After removing the red blood cells with an ACK buffer (pH 7.4), the splenocytes were washed three times in Ca^{2+}-Mg^{2+}free Hanks balanced salt solution and resuspended in RPMI1640 with 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 10% FBS. The total concentration of cells was $5X10^6$ cells/ml.

Cytotoxicity assay

The spleen cells were treated with LPS (50 μg/ml), Con A (10 μg/ml), LPS/ linarin (2.5, 5, 10, 20, 40 μg/ml), Con A/ linarin (2.5, 5, 10, 20, 40 μg/ml), linarin (2.5, 5, 10, 20, 40 μg/ml), all which were treated with Dulbeccos modified Eagle Medium(DMEM). They were instilled into each well $2X10^5$ cells/200 μl and were cultured in a CO_2 incubator (37°C, 5.5% CO_2) for 3 days. A cell Titer 96 Aqueous Non-Radioactive Cell Proliferation Assay Kit II (XTT: Promega, WI, USA) were used to measure the cytotoxicity on the spleen cells. Subsequently, 50 μl XTT reagent were added to each well, which were then cultured under the same conditions for 3 hours and measured by an ELISA reader at 490nm.

Cell culture

The mouse macrophages cell line (RAW 264.7), the IL-1 dependent cell line (D10), the TNF-α sensitive cell line (WEHI-164) were obtained from the American Type Culture Collection (ATCC). The cells were cultured in DMEM, which was supplemented with a high glucose, L-glutamine, 110mg/L sodium pyruvate, 10% fetal bovine serum (FBS), and 1% (v/v) penicillin (10000 U/ml)/ streptomycin (10000 U/ml)(P/S). The LPS (*Escherichia coli*, 0127: B8 Westphal type) was purchased from the Sigma Chemical Co. (St. Louis, MO. USA). The recombinant murine IL-1 (specific activity $1\text{-}2 \times 10^8$ U/mg) was purchased from R&D Systems (Oxon, UK).

Cytokines bioassay

The RAW 264.7 cells were cultured in DMEM with 10% FBS in 24-well-flat plates at a density of 5×10^5 cells/well. The cells were treated with different linarin concentrations (0, 2.5, 5, 10, 20, 40 μg/ml) and LPS 100 ng/ml in 5.5% CO_2 humidified air for 48 hours at 37°C. The supe-rnatants were then collected for cytokines bioassays.

IL-1 assay

In order to measure the IL-1 activity, the ability of the stimulated monocyte supernatants to cause proliferation of the IL-1 dependent T-cell line was assessed. An example was the conalbumin-spectific D10 G4.1 murine T-cell clone (ATCC), which requires IL-1β (1 u/ml) as well as specific antigens for growth. The IL-1 production assay was carried out according to the Manual of Clinical Laboratory Immunology (Noel *et al.*, 1991). The cells with IL-1β (1 u/ml) and those without in different culture supernatants of linarin (5, 10, 15, 20, 25 μl) were prepared as the treated groups. 8 wells per group were used and 100 μl of the cell suspension(4×10^4 cells/well) were added to each well and incubated in humidified air with 5.5% CO_2 for 48 hours 37°C. Subsequently, 50 μl of the XTT reagent was added to each well, cultured under the same conditions for 3 hours. They were then measured by an ELISA reader at 490 nm.

TNF-α assay

In order to investigate the TNF activity, the ability of the stimulated monocyte supernatants to lyse a highly TNF-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 linarin (5, 10, 15, 20, 25 μl) treated groups. 8 wells per group were used and 100μl of the WEHI-164 (2×10^4 cells/well) cells were added each well. The plates were then incubated in humidified air with 5.5% CO_2 for 48 hours at 37°C. Subsequently, 50μl of the XTT reagent was added to each well, cultured under the same conditions for 3 hours. The wells were then measured by an ELISA reader at 490 nm.

Western blot analysis of TNF-α

The cells were washed with phosphate buffered saline and lysed by boiling with a lysis buffer (1% SDS, 1.0 mM sod, vanadate, 10 mM Tris, pH 7.4) for 5 min. 35 μg protein from the cell lysates was applied to 15% SDS-polyacrylamide gels and transferred to a nitrocellulose (NC) membrane using the standard method (Choi *et al.*, 2000). The membrane was blocked with a 5% BSA solution for 1 hour. It was then incubated with anti-TNF-α monoclonal antibodies as the primary antibodies for 2 hours and washed 3 times with phosphate buffered saline. After incubating them with alkaline phosphates-labeled anti-antibodies for 1 hour, the bands were visualized by a Western Blot Kit substrate for phosphatase (LumiGLO System, KPL, U. S. A.).

Nitric oxide assay

Flat-bottomed 96 well, LPS (10 ng/ml), LPS/ linarin (2.5, 5, 10, 20, 40 μg/ml), Media only(DMEM-10), RAW 264.7 cell line, and Griess reagent (stock-I:0.2% naphylendia HCl, stock-II: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, cells only (1×10^6 cells/ml), LPS/ linarin (2.5, 5, 10, 20, 40 μg/ml), were prepared as the treated groups. 6 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 at room temperature and was measured by an ELISA reader at 540nm. Standard calibration curves were

prepared using sodium nitrite as the standard.

Cell staining

To determine the effects of linarin on the macrophage morphology, the cells were cultured in sterile glass-slide chambers at a density of 1000 cells/well for 48 hours. The culture medium was removed, and the cells were treated with either LPS (100 ng/ml), LPS/ linarin (2.5, 5, 10, 20, 40 μg/ml) or linarin (2.5, 5, 10, 20, 40 μg/ml) only for 2 days. Following the treatment, the culture supernatant was removed. Cells were fixed and stained in a Diff-quick Solution.

Flow cytometry

The RAW (3×10^5 cells/ml) cells were cultured in DMEM for 2 days. The media were changed and the cells were then incubated in the presence of either LPS (100 ng/ml), LPS/ linarin (2.5, 5 μg/ml) or the medium for 2 days. The treated cells were then scraped into PBS-0.1% sodium azide with 1% FBS (PBS-washing buffer, pH 7.2) and washed twice in a washing buffer at 4°C. Before staining the cells with FITC (Fluorescein isothiocyanate)-monoclonal antibodies, the cell surface Fc receptors were blocked by incubating the cells with 20μg/10^6 cells of purified anti-CD-16 for 30 min at 4°C. The residual antibodies were removed by washing. Anti-IA^b-FITC, Anti-CD80-PE (Phycoerythrin), Anti-CD86-FITC 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 fluores-cence intensity was determined on the 10,000 cells from each sample using logarithmic amplification.

Statistical analysis

Nitrite and cytokines production is expressed as a mean ± S.D. of 2 to 6 independent experiments. The statistical significance was determined using a Student-t tests.

RESULTS

Cytotoxicity of linarin against lymphocyte

In order to investigate the effect of linarin on lymphocyte proliferation, the splenocytes were incubated with either LPS, mitogen from B-lymphocytes, ConA, or mitogen from T lymphocytes, in the presence of various linarin concentrations. In this assay, Con A was added to the splenocytes at a concentration of 10 μg/ml. Linarin alone induced minimal lymphocyte proliferation (data not shown),

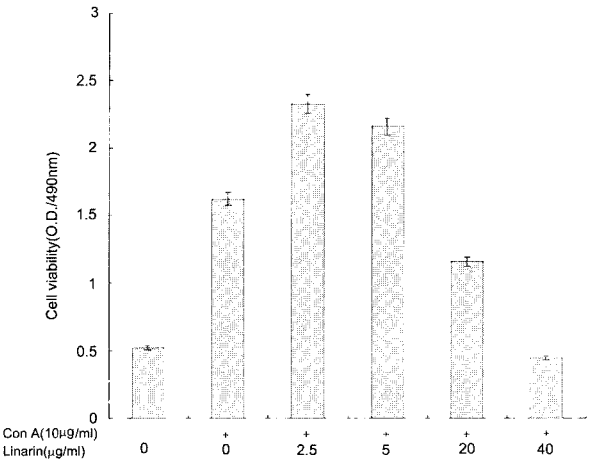

Fig.3. Inhibitory activity of linarin, a methanol extract from *Chrysanthemum zawadskii zawadskii* var, *latilobum* on Concanavallin A (ConA : 10 μg/ml)stimulated spleen cells. Treatment with various linarin concentrations (2.5, 5, 20, 40 μg/ml) with the Con Astimulated spleen cells. Using 8 wells per group, the cells were incubated for 3 days at 37°C in 5.45 % CO_2 after adding 50μl of the XTT labeling mixture. They were then incubated for 3 hours at 37°C in 5.45% CO_2. The absorbance was read using an ELISA reader at 490nm.

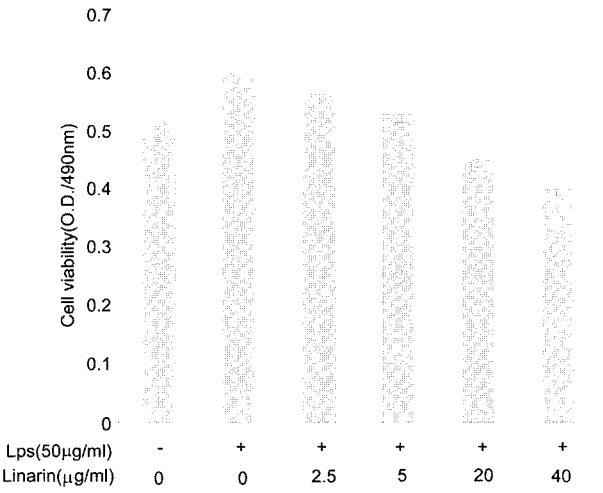

Fig. 4. Cytotoxic effect of linarin on total lymphocytes from spleen of BALB/c mice. The cells were purified from the spleen. The cells were treated with linarin and LPS at a concentration of 50 μg/ml LPS and various linarin concentrations (0, 2.5, 5, 20, 40 μg/ml) and incubated for 3 days at 37°C in 5.45% CO_2. After adding 50 μl of the XTT labeling mix-ture and incubating the cells for 3 hours at 37°C and 5.45% CO_2 the absorbance was read using an ELISA reader at 490nm.

Con A or LPS stimulated more lymphocyte proli-feration, and much greater proliferation was observed by the combined action of linarin and Con A or LPS. The proliferation of the T lymphocytes treated by Con A was significantly higher at 2.5 μg, than when treated with 5 μg/ml Con A. However, the number of total lymphocytes decreased with decreasing linarin concentrations (20 to 40 μg/ml), as shown in Fig. 3. The phase of the LPS

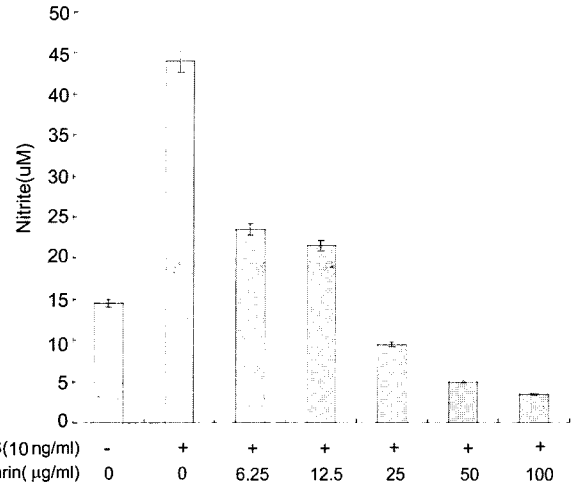

Fig. 5. Inhibitory effects of linarin on NO production in the LPS (lipopolysaccharide)-stimulated RAW 264.7cells. The cultures were incubated with 10 ng/ml of LPS in the presence of different linarin concentrations (6.25, 12.5, 25, 50,100 μg/ml). 6 wells per group were used and 200 μl of the cells were added to each well. The plates were then incubated overnight and 100 μl from the surface of each well was transferred into a new plate. The accumulated nitrite concentration were measured overnight after stimulation used a Griess reagent for 10 min at room temperature and read using an ELISA reader at 540 nm. NO production was determined by the accumulation of nitrite using the method reported Stuehr and Nathan (1989). The results are reported as a mean ± S.D. of three independent experiments.

stimulated lymphocytes also followed the same trend (Fig. 4). When the cells were treated with high concentrations of linarin (20 to 40 μg/ml) cytotoxicity in the splenocytes was observed.

Effect of linarin on RAW 264.7 cell NO Production

As shown in Fig. 5, the macrophages did not release NO in response to the medium. In this study, LPS was used as a positive control for macrophage activation. In the LPS (10 ng/ml) stimulated RAW 264.7 cell culture system, NO production was increased by i-NOS. When various linarin concentrations were added to the culture media at the time (40 hrs) cell stimulation, NO production was inhibited in a dose-dependent manner. Significantly, more NO was produced when the cells were exposed to a combination of LPS (10 ng/ml) and linarin (6.25, 12.5 μg/ml). From 25 to 100 μg/ml, a dose dependent decrease was observed. This is because one type of linarin character appeared to increase the cytotoxicity in the splenocytes at certain linarin concentrations (20 and 40 μg/ml). When a combination of LPS (10 ng/ml) and linarin (6.25, 12.5 μg/ml) were treated after inducing i-NOS pro-duction by 18 hours activation of the RAW 264.7

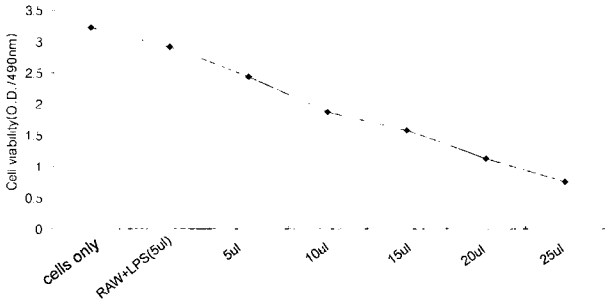

Fig. 6. LPS-induced TNF-α production by linarin in the mouse macrophage cell line. The RAW 264.7cells (5 X10^5 cells/ml) were cultured with the indicated linarin doses (2.5, 5,10, 20, 40 μg/ml) for 48 hours in the presence of 100 ng/ml LPS. The TNF-α bioassay was measured in the TNF-α sensitive cell line, WEHI-164, by their proliferation. The supernatant with the LPS-induced TNF-α production in the RAW cell was added to the TNF-α sensitive cell line, WEHI-164. The results are reported as a mean ± S.D. of three independent experiments.

cells, NO production was inhibited by 55.5%-65% compared to the LPS-control. Therefore, the concentrations required to inhibit NO production were calculated on the basis of concentrations of nitrite released into the culture media, as shown in Fig. 5. These results suggest that linarin may function, at least in part, through the macrophage activation in the host defense response.

Linarin alone did not affect NO production on the macrophage cell line (data not shown).

Direct cytokine production in response to linarin

TNF-α is one of the important proinflammatory cytokines that are mainly produced by monocytes and macrophages. To determine whether linarin (10 μg/ml) had a direct effect on cytokines production, a biological assessment of TNF-α activation was measured using the macrophage cell line. The proliferation assay was done by using TNF-α sensitive cell line WEHI 164, which will die with the treatment of culture soup containning TNF-α. As shown in Fig. 6, TNF-α production was increased by the linarin in the presence of LPS in a dose dependent manner. Western blot analysis (Fig. 7) showed that TNF-α production occurred after linarin treatment with the LPS

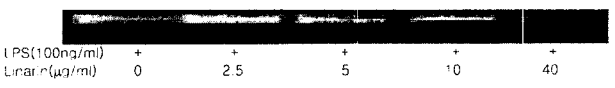

Fig. 7. Western Blot Analysis of the TNF-α in lysates of RAW 264.7 cells (35 μg Protein/Lane). The untreated cells and the cells incubated with LPS (100 ng/ml) alone and in combination with either linarin (2.5, 5, 10, 40 μg/ml) were separated by SDS-PAGE, transferred to a nitrocellulose and blotted with a mouse monoclonal anti-TNF-α antibodies.

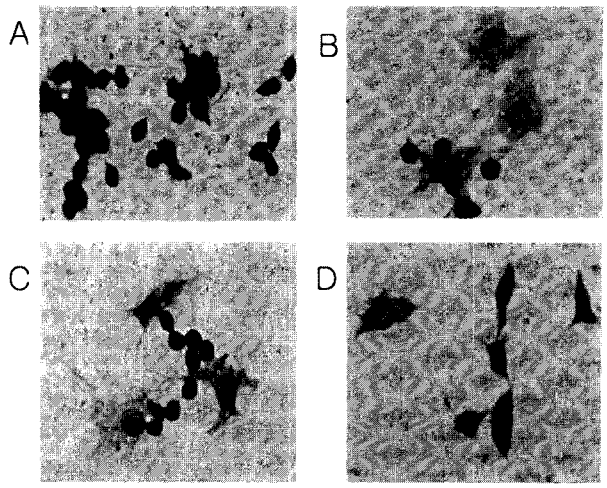

Fig. 8. Macrophage morphological changes in response to linarin. The RAW cells were cultured on cover slips in the presence of different concentration of the medium (A), linarin (2.5μg/ml)/LPS (100 ng/ml)(B), linarin (5 μg/ml)/LPS(C), linarin(10 μg/ml) / LPS(D) and LPS (100 ng/ml: data not shown) for 48 hours. The cells were fixed and stained in Diff-quick Solution.

(100 ng/ml). Linarin had a synergic effect on TNF-α production when treated with LPS. Linarin alone had no effect on macrophage activation according to western blot analysis (data not shown), LPS stimulated more macrophage activation, and much greater TNF-α production was generated by the combined action of linarin (2.5 μg/ml) and LPS. However, IL-1 production was not affected by linarin, as measured using a proliferation assay of an IL-1 dependent cell line (data not shown).

Effect of linarin and LPS on macrophage morphology

Normal RAW cells, when cultured in medium alone, tended to be round. None appeared to spread over the surface (Fig. 8A). Fig. 8 shows that the cells treated with linarin (2.5 μg/ml)/LPS (Fig. 8B) were larger and rougher than those exposed to either linarin (5 μg/ml)/LPS (Fig. 8C) or linarin (10 μg/ml)/LPS (Fig. 8D).

Effects of linarin on surface molecule expression

Since the adhesion molecules play an important role in the macrophage activation process. The RAW cell surface expression of I-A^b, CD80 (B7-1), and CD86 (B7-2) were examined by flow cytometry (Fig. 9). The major T-cell co-stimulatory molecules are B7-1 and B-2, which are closely related members of the immunoglobulin gene superfamily. They are expressed differentially on various agent presenting cell types, and may have different consequences for the responding T cells. The B7 molecules refers to both B7-1 and B-2. However, the surface I-A^b molecules were not expressed on the resting cells. The I-A^b molecules were

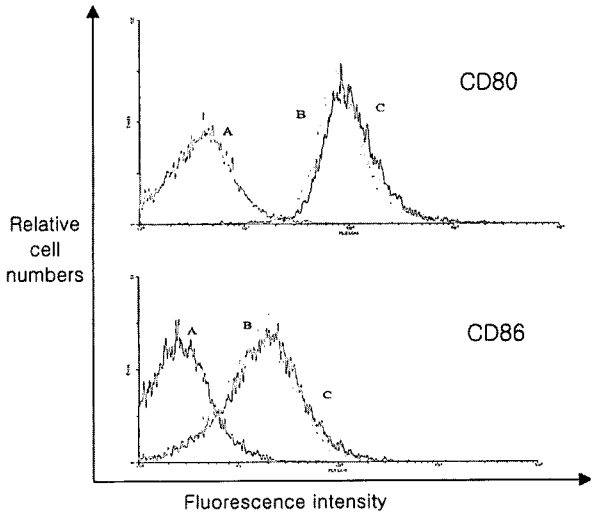

Fig. 9. Expression of the co-stimulatory molecules CD86, CD80, IA^b expression. The RAW cells were cultured in the presence of medium (A), LPS (100 ng/ml: data not shown), LPS/ linarin (2.5 μg/ml)(B), LPS/ linarin (5 μg/ml)(C) for 48 hours. The surface CD 80, CD 86, IA^b molecules were labeled with either anti-CD86-FITC, anti-CD80-PE, anti-IA^b FITC and the were cells stained using anti- Vβ8.1+8.2-FITC, anti-Vβ2-PE, anti-Vβ2-FITC, 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.

not expressed after exposure to linarin (not data shown). The surface CD80 and CD86 mole-cules were not expressed on the resting cells. However, Increased expression of CD80 and CD86 were observed on the cells treated with linarin (2.5, 5 μg/ml)/LPS (100 ng/ml).

DISCUSSION

In summary, none of the cells appeared to spread over the surface. The cells treated with LPS/linarin (2.5 μg/ml) were larger and rougher than those exposed to LPS/ linarin (5 and 10 μg/ml). This study showed the ability of linarin to activate macrophages both directly or indirectly and affecting both cytokine production and nitric oxide inhibition, in addition to the expression of some surface molecules. TNF-α production was dependent on the linarin dose. Linarin, a compound from *Chrysanthemum zawadskii* var, *latilobum* and its methanol extract, inhibited NO production in LPS-activated RAW 264.7 cells. Linarin may be a useful candidate as a new drug to treat endotoxemia and the inflammation accompanied by NO overproduction.

Activated macrophages fuse their lysosomes more efficiently to phagosomes, exposing intracellular or recently ingested extracellular microbes to a variety of microbicidal lysosomal enzymes. Activated macrophages

also produce oxygen radicals and nitric oxide (NO), both of which have potent antimicrobial activity, in addition to synthesizing antimicrobial peptides and proteases that can be released to attack extracellular parasites. Additional changes in activated macrophages assist in amplifying the immune response. The number of MHC class molecules, B7 molecules, and CD40 and TNF receptors on the macrophage surface increases, which causes the cell to be more effective at presenting an antigen to fresh T cells. These may therefore be recruited as effector cells, and be more responsive to both a CD40 ligand and to TNF-α.

NO is a multi-functional mediator, which plays an important role in regulating various biological functions *in vivo*. NO production by iNOS in macrophages is essential for the defense mechanisms against microorganisms and tumor cells (Faris *et al.*, 1994; MacMicking *et al.*, 1995). The NO is produced by iNOS, and mice that have had the gene for iNOS knocked out are highly susceptible to infections with several intracellular pathogens (Charles *et al.*, 1999). NO exhibits cytoprotective properties to some cells such as hepatocytes and endothelial cells (Kim *et al.*, 1997). However, its excessive production in inflammation is thought to be a causative factor for septic shock, cellular injury and carcinogenesis (Wolfe *et al.*, 1995). Therefore, the dysregulation of NO production is causatively related to the pathogenesis of various diseases, which include inflammation, cancer, immunological disorders, and vascular diseases. The inhibitory effect on NO production from activated macrophages has been demonstrated in some crude drugs with anti-inflammatory properties. Linarin, which inhibits i NOS expression, may have potential for treating endotoxemia and the inflammation that accompanies NO overproduction.

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 is produced by T_H 1 cells, some T_H2 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). T_H2 cells express B-cell-activating effectors molecules, whereas T_H1 cells express effector molecules that activate macrophages. TNF-α production was dependent on the linarin dose.

IL-1 is a multifunctional cytokine that is responsible for mediating a variety of processes in the host defense response, inflammation, and the response to injury. Macrophages and many other cell types produce IL-1 by the actions of various stimuli such as viruses, lipopolysaccharides, and phorbol esters. Reactive oxygen and nitrogen species are commonly produced during the inflammatory response and are involved in inflammatory cytokine production (Gossart *et al.*, 1996). Since IL-1 is a highly proinflammatory cytokine, agents that reduce its production and/or activity might be of a particular pharmacological and clinical interest.

Linarin was found stimulate macrophages to release TNF-α. NO production, cell morphological changes and surface molecule expression were largely unaffected by linarin alone. However, these effects could be potentiated by LPS addition. Therefore, these results demonstrate the ability of linarin to activate RAW cells both directly for cytokine production and indirectly for NO production and stimulate the expression of some of the surface molecules.

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