Anti-inflammatory Effect of LFR
on LPS-stimulated THP-1 Cells

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ABSTRACT

Background and Objective : Luffae Fructus Retinervus (LFR) is used for investigating symptoms of inflammation. We have evaluated the anti-inflammatory effect of LFR by analyzing the expression of pro-inflammatory cytokines.

Materials and Methods : We differentiated THP-1 cells into macrophage-like cells by treatment with PMA. Inflammation was induced by treatment with LPS and PMA. We determined the safe concentration of LFR by using the MTS and MTT assays and using PD98059 as a negative control for comparison of the anti-inflammatory effect of LFR.

Results : The MTS and MTT analysis showed that the cell survival rate was >80% within the LFR concentration range of 10-100 ng/ml and began to decrease to <80% at 1 μg/ml. By RT-PCR analysis, the gene expression of TNF-α, IL-8, TGF-β, IL-6, IL-1β, and IL-10 levels were down-regulated when monocyte-derived macrophages were treated with concentrations of LFR between 10 ng/mL and 100 ng/mL.

Conclusion : We conclude that LFR exerts an anti-inflammatory effect by inhibiting the expression of pro-inflammatory activity. The results suggest a promising way to treat general inflammatory diseases.

Key words : Luffae Fructus Retinervus, TNF-α, TGF-β, , IL-6, IL-10

Ⅰ. Introduction

Inflammation is a biological defense that removes or dilutes the causative factors for cellular injury. Inflammation is a complex response that occurs in vascularized connective tissue. The inflammatory response is a beneficial defense mechanism that restores damaged tissues, but it sometimes causes detrimental outcomes to the body[1].

The most important factor for inflammatory responses involves macrophages presenting antigens to T-lymphocytes in the recognition and activation stage of adaptive immunity. Macrophages also

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play a role as accessory cells that produce the membranous and secretory proteins for activation of T-lymphocytes. This accessory function of macrophages is enhanced by cytokines when microorganisms are encountered or during innate immunity against microorganisms[1].

Luffae Fructus Retinervus (LFR) has been used to treat inflammatory diseases in the field of Oriental medicine. There has been a few studies showing that herbal medicine has anti-inflammation effects, using LPS-stimulated THP-1 cells and and inflammation related cytokines. But the only studies that have been published about LFR are those using herbal-acupuncture[2,3,4,5].

Given the above background, we administered an extract of LFR to experimentally demonstrate the anti-inflammatory effectiveness of LFR in macrophages in which inflammation was induced by LPS. Then, we measured and analyzed changes in representative pro-inflammatory cytokines, such as IL-1β, IL-6, IL-8, and tumor necrosis factor (TNF)-α, and pro-fibrotic cytokines, such as transforming growth factor (TGF)-β, and anti-inflammatory cytokines, such as IL-10, using a reverse transcriptase polymerase chain reaction (RT-PCR).

II. Materials and methods

1. Preparation of the LFR extract

The LFR which was used in the current study was purchased from Kyounghee University School of Oriental Medicine Hospital. LFR (100 g) was placed in a vessel containing sterilized water and heated for 2 h. The LFR extract was collected and then concentrated using a reflux concentrator. The LFR extract was frozen at -80°C for 24 h.

Through a freeze drying process (Eyela, Japan), the dehydrated LFR extract was 10 g (yield = 10%).

To examine the effect of LFR on cell survival, cell viability was analyzed.

2. Cell culture conditions

The human monocytic leukemia cell line, THP-1, was obtained from the Korean Cell Line Bank (KCLB; Seoul, Korea). Cells were prepared and resuspended in RPMI 1640 medium (Gibco, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Gibco), and 1% antibacterial antifungal solution (Gibco). The cells were maintained in a humidified incubator (Sanyo, Japan) at 37° C in a 5% CO_2 + 95% O_2 atmosphere.

3. PMA treatment

THP-1 cells were differentiated in phorbol 12-myristate 13-acetate (PMA, 160 nM; Sigma, St. Louis, MO, USA) in RPMI medium for 72 h at 37°C., as determined in preliminary dose-response experiments. The criteria for the differentiation of THP-1 cells were adherence, changes in cell morphology, and changes in the profile of cell surface maker expression (integrin, FcγRI, CD4, and MHC class II antigens). The PMA solution was prepared by dissolving PMA in sterile dimethylsulfoxide (DMSO) (Sigma). The stock solution was stored at -20° C. Immediately prior to use, the PMA stock solution was diluted in RPMI medium to 160 nM.

4. Macrophage differentiation and stimulation

THP-1 cells were seeded onto macrophages (1 x 10^6 cells) in 100 mm^2 dishes for experiments. The cells were pretreated with 160 nM of PMA for 72

h and subsequently stimulated with 5 μg/ml of LPS from *E. coli* for 2 h in a humidified incubator (Sanyo) at 37° C in a 5% CO_2 + 95% O_2 atmosphere. PMA and LPS were dissolved in DMSO and RPMI 1640 medium.

5. Reagents

The following reagents were used in the experiments herein: PMA, 5 μl/50 ml (5μg/ 50 ml) media (P1585 – 1 μg, dissolved in DMSO); polymycin B, 0.1 μl/ml media (50 mg/ml); LPS (1 mg/ml), 5 μl/ml or (5 μg/ml) media; LFR extract, (10 ng and 100 ng/ml) media; polymycin B, stock (50 mg/ml)à(0.1 μl/ml) à 5 μg/ml of media; and PD098059, (dissolved in DMSO) 100 μM à 106.92 μl (1 mg/ml).

6. Cell viability assay

Cell viability was determined by using a 3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethonyp henol)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) kit (Promega, Madison, WI, USA and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide(MTT) assay, according to the manufacturer's protocol (Promega). For analysis of cell toxicity by the LFR extract, cells were pre-treated with PMA for 72 h and treating the LFR extract at 1-1000 ug for 3 h. The control group was treated with the same amount of PBS and the MTS and MTT labeling reagents (20 μl) were added to each group and incubated for 2 h at 37° C. The absorbance of the wells was measured at 490 and 670 nm using a Soft Max ELISA (Molecular Devices, USA). The optical density (OD) was calculated as the difference between the reference wavelength and the test wavelength. The percent cell viability = (A490 or A670/nm of drug-treated cells/A490or A670/nm of control cells) x 100.

7. Experiment

This experiment studied the expression of cytokines by RT-PCR methods for the analysis of the level of mRNA. For analysis of the anti-inflammatory effect of the LFR extract, we determined the expression of inflammation-related cytokine mRNA by RT-PCR analysis. After THP-1 cells were exposed to PMA for 72 h, the cells were treated with LPS and LFR extract at the same time. At this point, the TNF-α pathway inhibitory drug (PD 98059) was used as a negative control.

8. RT-PCR analysis

Total cellular RNA was isolated from THP-1 cells using the Trizol reagent (Gibco). The cDNA was synthesized from 1 μg of RNA using 200 U Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen, Carlsbad, CA, USA) in a total reaction volume of 20 μl. The cDNA was amplified using gene-specific primers. Amplified products were analyzed using an Image Documentation System (GelDoc 2000; Bio-Rad, USA) with image analysis software (Quantity One; Bio-Rad). DNA size markers (Fermentas, USA) were run in parallel to validate the predicted sizes of the amplified bands. The primer sequences are listed in Table 1.

9. Statistical Analysis

The values are expressed as the mean ±S.E.M. The differences between the groups were determined by one-way ANOVA, followed by Tukey's HSD test for all pairwise multiple comparisons. The following P values were considered statistically significant: *, P<0.05; **, P<0.01; and ***, P<0.001.

Table 1. Primer sequences and PCR product sizes

Name		Oligonucleotide sequence (5'-3')	Product size(bp)
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	sense	GGTGAAGGTCGGAGTCAACGG	500[a]
	anti-sense	GGTCATGAGTCCTTCCACGAT	
Interleukin-1β (IL-1β)	sense	GGGCCTCAAGGAAAAGAATC	470[b]
	anti-sense	AGCTGACTGTCCTGGCTGAT	
Interleukin-6 (IL-6)	sense	AAAGAGGCACTGGCAGAAAA	408[b]
	anti-sense	GAGGTGCCCATGCTACATTT	
Interleukin-8 (IL-8)	sense	AGGGTTGCCAGATGCAATAC	378[c]
	anti-sense	AGACTAGGGTTGCCAGA	
Tumor necrosis factor-α (TNF-α)	sense	AGCCCATGTTGTAGCAAACC	424[d]
	anti-sense	CCAAAGTAGACCTGCCCAGA	
Transformation growth factor-β (TGF-β)	sense	GACTGCGGATCTCTGTGTCA	480
	anti-sense	CTGGTCTCAAATGCCTGGAT	
Interleukin-10 (IL-10)	sense	AAGCCTGACCACGCTTTCTA	463
	anti-sense	TTCCATCTCCTGGGTTCAAG	

a) Dixon et al., 1999; b) Glue et al., 2002; c) Na et al., 2001; d) de Groot- Kruseman et al., 2003

III. Results

1. Anti-inflammatory effects of the LFR extract on THP- 1 cells

Cell viability was measured using the MTS and MTT assays. As a result, macrophages, which were differentiated into white blood cells had a cell viability of >80% with LFR (10 ng/ml). However, the cell viability was noted to markedly decrease at a concentration of >1 μg/ml (Figs. 1 and 2).
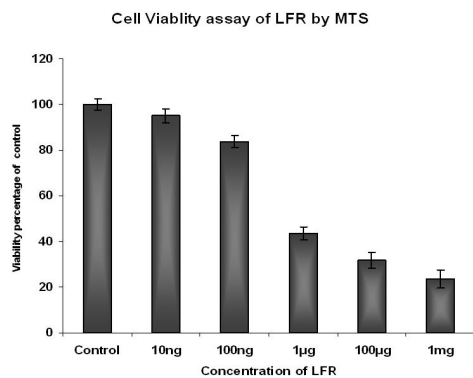

Cell Viablity assay of LFR by MTS

Fig. 1. Effects of LFR on cell viability of THP-1

cells after 72 h of PMA activation by the MTS assay.

Values represent the % of control. Experiments were performed in triplicate and each value is the mean of three independent experiments.
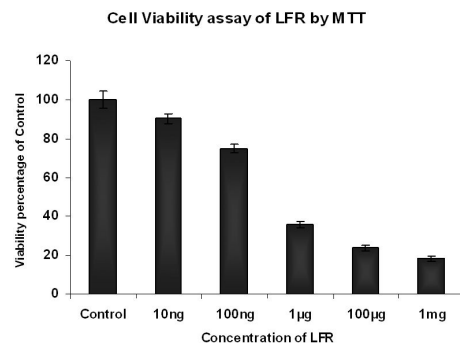

Cell Viability assay of LFR by MTT

Fig. 2. Effects of LFR on cell viability of THP-1 cells after 72 h of PMA activation by the MTT assay.

Values represent the % of control. Experiments were performed in triplicate and each value is the mean of three independent experiments.

2. Transcriptional activities of cytokines by the LFR extract

To examine the anti-inflammatory effect of LFR, LFR and LPS were synchronously treated. The degree of the inhibited expression of cytokines was measured using RT-PCR. A comparison with PD 98059 was also made. LFR had a cytotoxic effect at concentrations >1μg/ml. The current experiment was therefore performed at two concentrations (10 ng/ml and 100 ng/ml).

3. The effect of LFR on the expression of TNF-α and IL-8 mRNA

The expression of TNF-α and IL-8 mRNA was suppressed. The expression of TNF-α mRNA was significantly suppressed at both concentrations (10 ng/ml [$p < 0.001$] and 100 ng/ml [$p < 0.001$]). The expression of IL-8 mRNA was significantly suppressed only at a concentration 100 ng ($p < 0.001$; Fig. 3 and 4)
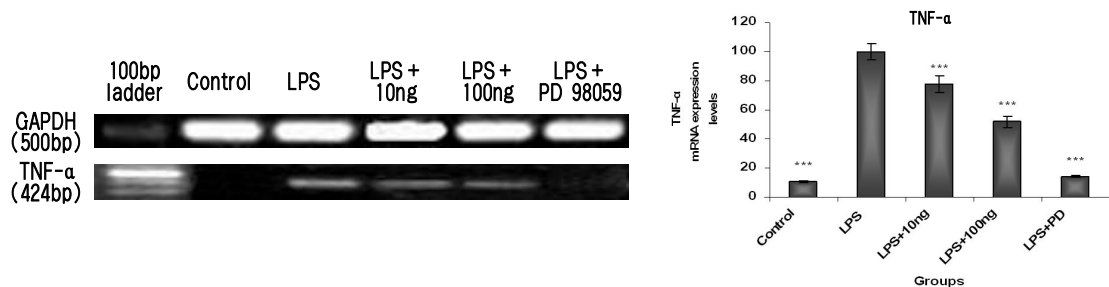

Fig. 3. Transcriptional activities of TNF-α.

Control: non-treated group, LPS: PMA and LPS-treated group, LPS+10 ng : LPS and LFR-treated (10 ng/ ml) group, LPS+100 ng: LPS and LFR-treated (100 ng/ml) group, LPS+ PD : LPS and PD 98059-treated group. Each of the PCR products was analyzed against GAPDH. The observed bands were correlated with the predicted size for GAPDH (500 bp) and TNF-α (424 bp). Data were presented as the mean ±SEM. Statistical analysis was performed using one-way ANOVA. ***$p < 0.001$ compared to LPS.
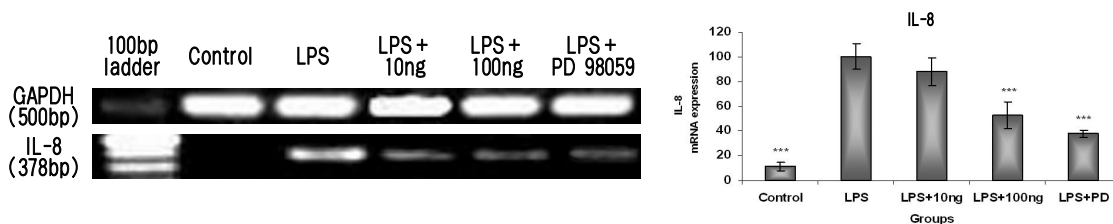

Fig. 4. Transcriptional activities of IL-8.

Control: non-treated group, LPS: PMA and LPS-treated group, LPS+10 ng : LPS and LFR-treated (10 ng/ ml) group, LPS+100ng : LPS and LFR-treated (100 ng/ml) group, LPS+ PD : LPS and PD 98059-treated group. Each of the PCR products was analyzed against GAPDH. The observed bands were correlated with the predicted size for GAPDH (500 bp) and IL-8 (378 bp). Data were presented as the mean ±SEM. Statistical analysis was performed using one-way ANOVA. ***$p < 0.001$ compared to LPS.

4. The effect of LFR on the expression of TGF-β and IL-6 mRNA

The expression of TGF-β and IL-6 mRNA was suppressed. The expression of TGF-β mRNA was

significantly suppressed at both concentrations (10 ng/ml [p<0.01] and 100 ng/ml [p<0.001]). The expression of IL-6 mRNA was significantly suppressed

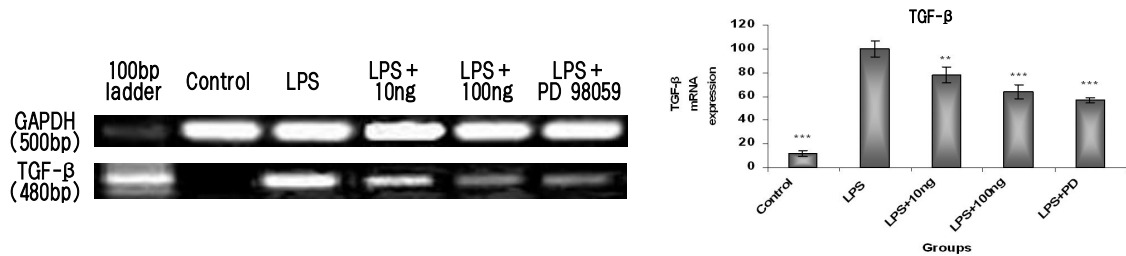at both concentrations (10 ng/ml [p<0.001] and 100 ng/ml [p<0.001; Figs. 5 and 6).

Fig. 5. Transcriptional activities of TGF-β.

Control: non-treated group, LPS: PMA and LPS-treated group, LPS+10 ng : LPS and LFR-treated (10 ng/ml) group, LPS+100 ng: LPS and LFR-treated (100 ng/ml) group, LPS+ PD : LPS and PD 98059-treated group. Each of the PCR products was analyzed against GAPDH. The observed bands were correlated with the predicted size for GAPDH (500 bp) and TGF-β (480 bp). Statistical analysis was performed using one-way ANOVA. **p<0.01, ***p<0.001 compared to LPS.
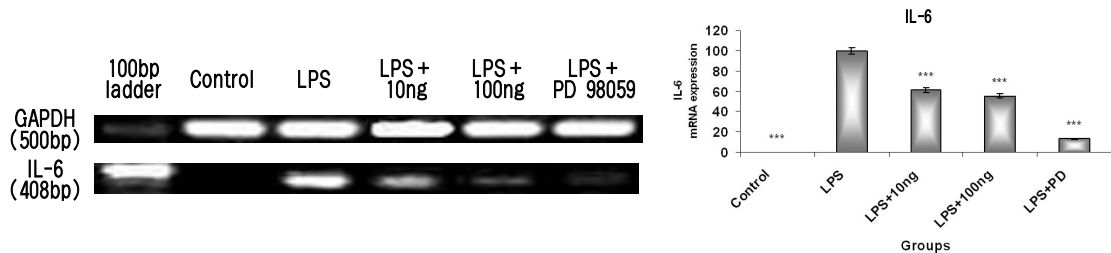

Fig. 6. Transcriptional activities of IL-6.

Control: non-treated group, LPS: PMA and LPS-treated group, LPS+10 ng : LPS and LFR-treated (10 ng/ml) group, LPS+100 ng: LPS and LFR-treated (100 ng/ml) group, LPS+ PD: LPS and PD 98059-treated group. Each of the PCR products was analyzed against GAPDH. The observed bands were correlated with the predicted size for GAPDH (500 bp) and IL-6 (408 bp). Data were presented as the mean ±SEM. Statistical analysis was performed using one-way ANOVA. ***p<0.001 compared to LPS.

5. The effect of LFR on the expression of IL-1β and IL-10 mRNA

The expression of IL-1β and IL-10 mRNA was suppressed. The expression of IL-1β mRNA was

significantly suppressed only at 100 ng/ml (p<0.001). The expression of IL-10 mRNA was significantly suppressed at both concentrations (10 ng/ml [p<0.001] and 100 ng/ml [p<0.001; Fig. 7 and 8).
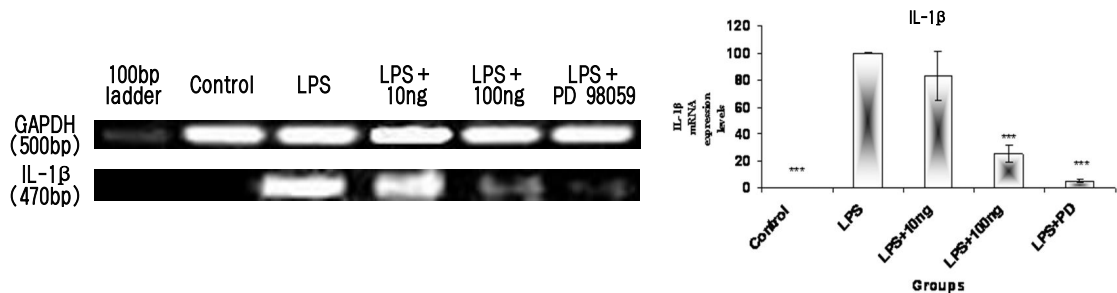
Fig. 7. Transcriptional activities of IL-1β.

Control: non-treated group, LPS: PMA and LPS-treated group, LPS+10 ng : LPS and LFR-treated (10 ng/㎖) group, LPS+100 ng : LPS and LFR-treated (100 ng/㎖) group, LPS+ PD : LPS and PD 98059-treated group. Each of the PCR products was analyzed against GAPDH. The observed bands were correlated with the predicted size for GAPDH (500 bp) and IL-1β (470 bp). Data were presented as the mean ±SEM. Statistical analysis was performed using one-way ANOVA. ***$p < 0.001$ compared to LPS.
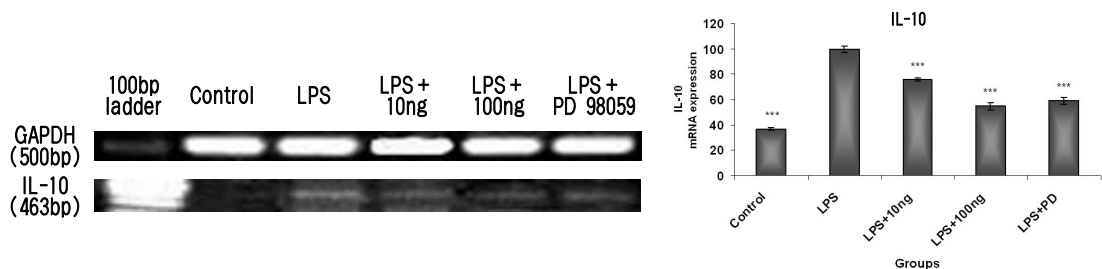

Fig. 8. Transcriptional activities of interleukin-10 (IL-10).

Control: non-treated group, LPS: PMA and LPS-treated group, LPS+10 ng: LPS and LFR-treated (10 ng/㎖) group, LPS+100 ng : LPS and LFR-treated (100 ng/㎖) group, LPS+ PD : LPS and PD 98059-treated group. Each of the PCR products was analyzed against GAPDH. The observed bands were correlated with the predicted size for GAPDH (500 bp) and IL-10 (463 bp). Data were presented as the mean ±SEM. Statistical analysis was performed using one-way ANOVA. ***$p < 0.001$ compared to LPS.

Ⅳ. Discussion

Luffae Fructus Retinervus (LFR) has been used to treat inflammatory diseases in the field of Oriental medicine. There has been a few studies showing that herbal medicine has anti-inflammation effects, using LPS-stimulated THP-1 cells and and inflammation related cytokines. But the only studies that have been published about LFR are those using herbal-acupuncture[2,3,4,5].

In this study, we demonstrated the close relationship between LPS-induced inflammation in THP-1 cells and production of inflammatory cytokines by PMA-treated THP-1 cells in LFR extract treatments. The endotoxin (lipopolysaccharide [LPS]) of gram-negative bacteria is believed to be responsible for initiating host responses leading to inflammation. LPS is not intrinsically toxic, but acts by inducing myeloid and/or non-myeloid cells to express a multiplicity of genes encoding proteins with activities that produce the hemodynamic and hematologic changes observed in inflammation.

LPS is one of the most potent biological response modifiers known; picomolar concentrations are sufficient to stimulate cells of the immune /inflammatory/vascular systems[6]. A vast amount of information about the molecular mechanism of host defense responses and inflammatory mediators has been derived from studies using LPS as a stimulus[7]. Recognition of LPS triggers gene induction by myeloid and non-myeloid lineage cells. These inducible genes encode proteins that include cytokines, adhesive proteins, and enzymes that produce low molecular weight pro-inflammatory mediators. Macrophages are potent effector cells that are able to engulf and kill bacterial invaders. Inflammation is a complex stereotypical reaction of the body expressing the response to damage of its cells and vascularized tissues. Macrophages are activated by innate immune receptors, such as CD14, which detect LPS, peptidoglycan, and other molecules displayed on the surface of microorganisms[8]. These cells play a central role in inflammation and the regulation of the immune response. LPS, a component of the cell wall of gram-negative bacteria, activates macrophages to produce pro-inflammatory cytokines, such as TNF-α and interleukin-1 (IL-1), and secondary mediators, such as leukotrienes and prostaglandins (PGs). These substances are important regulators of both innate and adaptive immunity[9]. However, their uncontrolled expression can cause acute or chronic inflammatory syndromes. An acute inflammatory syndrome induced by these mediators is the septic shock syndrome, which is characterized by fever, hypotension, disseminated intravascular coagulation, and multiple organ failure[10].

Currently, corticosteroids are the most common pharmacologic drugs to control inflammation in the clinic. But, these drugs have significant side effects, especially when it is used chronically. Therefore, there is tremendous interest in developing safer anti-inflammatory drugs. By using LPS as stimuli, this study observed the control mechanism(s) of inflammation. For comparison of anti-inflammatory effect(s) of the LFR extract, PD 98059 is used as a negative control. PD 98059 selectively blocks the activity of MEK, inhibiting both the phosphorylation and activation of MAP kinases *in vitro*. This inhibitor discovered by screening a chemical library for inhibitors of the MAP kinase cascade[11].

This study had an emphasis on THP-1 cells because the cells are a well-characterized human monocytic leukemic cell line; the cells resemble monocytes with respect to several criteria and can be differentiated into macrophage-like cells by treatment with PMA. PMA-induced differentiation is associated with alterations in cell morphology, adherence, and expression of several genes[12]. In this way, THP-1 cells are differentiated to examine the pattern of expression of cytokines. By using the MTS assay and MTT, the safety of the LFR extract was demonstrated. As a result, LFR extract did not affect the cell survival within 1-100ng/㎖ (Figs. 1 and2.). Therefore, the extract does not affect cell survival until a critical point.

Cytokines are usually extracellular signaling proteins, usually <80 KD in size, and many are glycosylated[13]. They are produced by many different cell types that are involved in cell-to-cell interactions acting through specific receptors on the surface of target cells. Cytokines usually have an effect on closely adjacent cells and therefore function in a predominantly paracrine fashion, although they may also act at a distance (endocrine) and may have effects on the cells of

origin (autocrine). Cytokines may be regarded as a mechanism for cell-cell communication, and within this group may be included growth factors and cytokines with primarily chemoattractant properties (chemokines). They act on target cells to cause a wide array of cellular functions, including activation, proliferation, chemotaxis, immunomodulation, release of other cytokines or mediators, growth and cell differentiation, and apoptosis. Cytokines were originally characterized (and named) according to some aspect of their functional activity that was initially discovered, but the cloning of the genes for these cytokines has now provided a better insight into their classification and grouping. It is apparent that there is a wide pleiotropy and element of redundancy in the cytokine family in that each cytokine has many overlapping functions, with each function[14]. Macrophages, one of the myeloid lineage cells, perform critical functions in the immune system. They act as regulators of homeostasis and as effector cells in infection, wounding, and tumor growth[15]. Macrophages originate in the bone marrow, and through the blood stream reach all the tissues in the organism. According to the specific needs, tissue macrophages proliferate, further differentiate into more specialized macrophage populations, or become activated[16]. In this study, we demonstrated the possibility of herbal medicine as an alternative treatment for inflammatory-related diseases and our research is focused on treatment for the inflammation to investigate LFR extract anti-inflammatory properties.

TNF-α is a pro-inflammatory cytokine that plays an important role in mechanisms of host defense against intracellular pathogens. Several bacterial components have been described that can affect the levels of TNF-α expression[17]. For the analysis of inflammation-induced difference we used LPS and PMA in THP-1 cells. The levels of TNF-α and mRNA was analyzed by RT-PCR. TNF-α is expressed in response to inflammatory stimuli, such as LPS. THP-1 cells are the macrophage branch of the myeloid lineage and can be induced by a variety of agents, including PMA, to differentiate from a promonocytic to a monocytic stage of development. According to previous studies, TNF-α mRNA is not induced by LPS unless the cells are differentiated by PMA on THP-1 cells. Eventually, treatment of PMA with LPS for 2 h caused substantial up-regulation of TNF-α mRNA levels in THP-1 cells, while non-treatment of PMA and LPS is not expressed the gene(Fig. 3).

Macrophages are potent effector cells that are able to engulf and kill many bacterial invaders. Several facultative intracellular bacteria[18] infect macrophages. The infected macrophages secrete various pro-inflammatory cytokines, such as IL-1, TNF-α, and IL-6 which alert other cells in the immune system. Pro-inflammatory cytokines lead to activation of macrophages and killing of intracytoplasmic bacteria. Generated cytokines also contribute to inflammatory tissue damage[18]. As a result, inflammation is regulated by the balance between pro-inflammatory (IL-1β, TNF-α, IL-6, and IL-8,) and anti- inflammatory (TGF-β and IL-10) cytokines.

This study examined the expression of pro-inflammatory cytokines to control the inflammation. For the analysis of anti-inflammatory effect in terms of the concentration of the LFRextract, the transcriptional activities of IL-1β, IL-6, IL-8, TNF-α, TGF-β, and IL-10 were down-regulated

more than the LPS-treated group (Figs. 3-8). Thus, it is possible to use LFR for the treatment of inflammatory diseases dependent on the dose of the LFR extract.

For analysis of the anti-inflammatory effect of the LFR extract, the expression of inflammation-related cytokines was determined by RT-PCR. After THP-1 cells were exposed to PMA for 72 h, the cell culture and LPS treatment(s) were started. As the concentration of LFR extract (10 ng/ml and 100 ng/ml), transcriptional activity of IL-1β decreased to 19% and 70%. In the case of transcriptional activity of IL-6, it decreased to 35% and 40%. The transcriptional activity of IL-8 also decreased to 10% and 50% against the LPS-treated group. The transcriptional activity of TNF-α was down-regulated to 22% and 45% against the LPS-treated group. The transcriptional activity of TGF-β was down-regulated to 20% and 40% against the LPS-treated group. Finally, the transcriptional activity of IL-10 was down-regulated to 25% and 43% against the LPS-treated group. In conclusion, all the cytokine levels were down-regulated by the LFR extract (concentrations up to 100 ng/ml) on monocyte-derived macrophages (Figs. 3-8), which indicates the LFR extract is involved in the inflammatory mechanism(s).

V. Conclusion

The expression of various cytokines was down-regulated by the MAP kinase inhibitor, PD098059, or LFR extract treatment of macrophages with LPS-induced inflammation(Fig. 3-8) as shown above. In LFR extract-treated cells, expression of TNF-α, IL-8, TGF-β, IL-6, IL-1β, and IL-10 mRNA were down-regulated compared to LPS-treated cells when compared to control. As a result, the LFR extract effectively suppressed inflammation-related cytokines on macrophages with LPS-induced inflammation.

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