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Anti-inflammatory effect of Samultang in human mast cell line HMC-1

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SUMMARY

Samultang has been believed for prevention and remedy various blood diseases such as menstrual irregularity, anemia, and metrorrhagia. However, the mechanism that accounts for anti-inflammatory effects of the Samultang is still not fully understood. This study was designed to evaluate whether and how the Samultang could modulate the production of pro-inflammatory cytokines in phorbol 12-myristate 13-acetate (PMA) plus calcium ionophore A23187 treated-human mast cell line, HMC-1. Samultang inhibited the production of tumor necrosis factor (TNF)-α, interleukin (IL)-6, granulocyte macrophage colony stimulating factor (GM-CSF), and vascular endothelial growth factor (VEGF) in HMC-1. Maximal inhibition rate of TNF-α, IL-6, GM-CSF, and VEGF by 0.1 mg/ml Samultang was about 70.73 ± 3.0%, 51.49 ± 4.14%, 54.03 ± 2.09%, and 47.95 ± 7.86%, respectively. Samultang partially blocked PMA plus A23187-induced cyclooxygenase (COX)-2 expression. In addition, Samultang inhibited activation of nuclear factor (NF)-κB, and extracellular signal-regulated kinase (ERK) activation. These results suggest that anti-inflammatory effect of Samulatng may be mediated by the suppression of cytokine production and COX-2 activation via down-regulation of NF-κB and ERK activation.

Key words: Samultang; Cytokine; COX-2; NF-κB; ERK

INTRODUCTION

Mast cells are widely distributed throughout the body in both connective tissue and at mucosal surfaces . The role of mast cells as effector cells of immunoglobulin E (IgE)-dependent immediate-type hypersensitivity reactions and anaphylaxis is well understood (Galli, 1993). Mast cells are also involved in the development of late-phase reactions and influence other chronic inflammatory responses through the generation and production of various cytokines (Kruger-Krasagakes and Czarnetzki, 1995). There are well known mast cells are a potential source of cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-4, IL-6, IL-8, and IL-13, granulocyte macrophage colony stimulating factor (GM-CSF), transforming growth factor-1, and vascular endothelial growth factor (VEGF) (Grutzkau *et al.*, 1998; Artuc, 1999; Bradding and Holgate, 1999; Stassen, 2001). The human mast cell line, HMC-1, should be a useful tool for studying cytokine activation in human mast cells (Sillaber *et al.*, 1993; Nilsson *et al.*, 1995).

Cyclooxygenase (COX)-2 plays important roles in various tumors and inflammatory diseases (Kong *et al.*, 2002). COX-2, one of the major mediators of the inflammatory reaction, is also strongly induced in activated monocytes/macrophages.

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Several recent studies demonstrated that prostaglandins, which is the COX-2 metabolite released from activated mast cells, is also essential for the pathogenesis of eosinophilic airway inflammation (Bochenek *et al.*, 2004).

Nuclear factor (NF)-κB may induce many inflammatory genes that encode for pro-inflammatory cytokines, chemokines that selectively attract inflammatory cells, inflammatory enzymes [such as inducible NO synthase (iNOS) and COX-2], adhesion molecules and inflammatory receptors (such as IL-2 receptors) (Barnes and Adcock, 1997). In most cell types, the pleiotropic-inducible form of NF-κB is a heterodimer composed of p50 and RelA/p65. NF-κB normally resides in the cytoplasm, where it is retained by association with IκB protein, an endogenous inhibitor (Barnes and karin, 1997). When NF-κB is activated, it translocates to the nucleus, and activates inflammatory involved genes (Palombella *et al.*, 1994).

MAPKs cascade is a major signaling pathway in many cells. In mammalian cells, three important groups of kinase pathways compose the MAPK family including the extracelluar signal-regulated kinases (ERK), the p38 MAPK, and the c-Jun NH2-terminal kinase (JNK). The ERK-cascade appears to mediate signals promoting cell proliferation, differentiation, or survival, whereas the p38 MAPK and JNK cascades appear to be mainly involved in cellular stress responses. And the induction of most cytokine genes requires activation of the ERK and p38 MAPK (Shapiro and Dinarello, 1995). The activated kinases initiate a cascade of protein phosphorylation involving multiple other kinases and activate nuclear transcription factors such as NF-κB, activated protein (AP)-1, and activating transcription factor (ATF) (Gupta *et al.*, 1996; Barchowsky *et al.*, 2000), which promote the expression of inflammatory cytokines and chemokines.

Samultang has been believed for prevention and remedy all kinds of blood diseases such as menstrual irregularity, anemia, and metrorrhagia. In a number of studies, Samultang has been investigated anti-stress, lucopenia, thrombocytopenia, anemia, and reducing side-effects of the anti-cancer agents. So *et al.* reported that Samultang protects the lipopolysaccharide/prorbol 12-myristate 13-acetate (PMA)-induced damage and nitric oxide production of C6 glial cells. They suggested that Samultang was clinically useful to treat the ischemic brain disease (So *et al.*, 2000). This study was designed to evaluate whether and how the Samultang could modulate the production of pro-inflammatory cytokines and COX-2 expression in PMA plus A23187 treated-HMC-1. Also, to find a possible explanation for the anti-inflammatory mechanisms of Samultang, we evaluate effects of Samultang on PMA plus A23187-induced NF-κB activation, and ERK acticvation in HMC-1.

MATERIALS AND METHODS

Preparation of Samultang

Samultang was prepared by decocting the dried prescription of samultang with boiling distilled water. The duration of decoction was about 150 min. The decoction was filtered, lyophilized and kept at 4°C. The Samultang water extract powder was dissolved in sterile saline. Samultang includes same weight (4.8 g) of Radix Rehmanniae Preparata, Radix Paeoniae Alba, Rhizoma Cnidii, and Radix Angelicae Gigantis, respectively.

Reagents

Fetal bovine serum (FBS), and Iscove's Modified Dulbecco's Medium (IMDM) were purchased from Gibco BRL (Grand Island, NY, U.S.A.). PMA, A23187, avidin-peroxidase, 2, 2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid), 3-(4, 5-dimethylthiazol-2-yl)-diphenyl -tetrazolium bromide (MTT), and other reagents were obtained from Sigma (St. Louis, MO, U.S.A.). Anti-human TNF-α/IL-6/GM-CSF/VEGF antibody (Ab), biotinylated anti-human TNF-α/IL-6/GM-CSF/VEGF Ab, and recombinant human (rh) TNF-α/IL-6/GM-CSF/VEGF were purchased from R&D Systems (Minneapolis, MN, U.S.A.). Abs for anti-

NF-κB, COX-2, phospho-specific ERK (pERK), pJNK, pp38, ERK, JNK, and p38 were purchased from Santa Cruz Biotech. Inc. (Santa Cruz, CA, U.S.A.).

Culture of HMC-1

HMC-1 was grown in IMDM supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated FBS at 37 in 5% CO_2 incubator.

MTT assay

To test the viability of cells, MTT colorimetric assay was performed as described previously (Kim *et al.*, 2004). Briefly, HMC-1 (1×10^6 cells/ml) were incubated for 24 h after stimulation in the absence or presence of Samultang (0.01 mg/ml, 0.1 mg/ml, and 1 mg/ml). After addition of MTT solution, the cells were incubated at 37°C for 4 h. The crystallized MTT was dissolved in dimethyl sulfoxide and measured the absorbance at 540 nm by ELISA reader (Versa Max, Molecular devices).

Enzyme-linked immunosorbent assay (ELISA)

TNF-α, IL-6, GM-CSF, and VEGF production was measured by modification of an ELISA as described previously (Kim *et al.*, 2004). HMC-1 cells were cultured with IMDM plus 10% FBS. The cells were sensitized with PMA (50 nM) plus A23187 (1 μM) for 8 or 24 h in the absence or presence of Samultang. The ELISA was performed by coating 96-well plates (Nunc, Denmark) with 1 g/well of murine monoclonal Ab with specificity for TNF-α, IL-6, GM-CSF, and VEGF. Before use and between subsequent steps in the assay, the coated plates were washed twice with PBS containing 0.05% Tween - 20 (PBST). All reagents used in this assay and the coated wells were incubated for 1 h at room temperature. For the standard curve, rh TNF-α, IL-6, GM-CSF, and VEGF antibody was added to the wells. After exposure to the medium, the assay plates were exposed sequentially to biotinylated anti-human TNF-α, IL-6, GM-CSF, and VEGF. After 1 h, the assay plates were exposed to enzyme, avidin-peroxidase, for 30 min. And then substrate, 2, 2-azino-bis (3-ethyl benzthiazoline -6-sulfonic acid) tablet, was added to the wells. Optical density was read within 10 min of the addition of the substrate on a ELISA reader (VersaMax, Molecular Devices) with a 405 nm filter.

Western blot analysis

For detecting COX-2 and MAPKs, cell extracts were prepared by detergent lysis procedure. Cells (2×10^6 cells) were harvested, washed once with PBS, and resuspended in lysis buffer. Samples were vortexed for lysis for a few seconds every 15 min at 4°C for 1 h and centrifuged at 15,000 × *g* for 5 min at 4°C. Samples were heated at 95°C for 5 min, and cooled on ice followed by centrifugation at 15,000 × *g* for 5 min. 30 mg total proteins were loaded and separated on 10% SDS-polyacrylamide gels. After electrotransferring onto nitrocellulose membrane (Amersham Parmacia Biotech UK limited, England) at 4°C, the membrane was blocked with 5% nonfat dry milk in PBST for 1 h. After slightly washing with PBST, membrane was probed with primary Ab for 1 h and washed three times with PBST. Horseradish peroxidase-conjugated secondary Ab was incubated and chemiluminescence detection was performed using ECL detection reagent (Amersham Parmacia Biotech UK limited, England). Proteins were visualized by fluorography using Agfa X-ray film blue.

Nuclear protein extraction

After cell activation for the times indicated, 1×10^7 cells were washed in 1 ml of ice-cold PBS, centrifuged at 1,000 × *g* for 5 min at 4°C, resuspended in 400 μl of ice-cold hypotonic buffer (10 mM HEPES/KOH, 2 mM $MgCl_2$, 0.1 mM EDTA, 10 mM KCl, 1 mM DTT, O.5 mM PMSF, pH 7.9), left on ice for 10 min, vortexed, and centrifuged at 15,000 × *g* for 30 s. Pelleted nuclei were gently resuspended in 50 ml of ice-cold saline buffer (50 mM HEPES/KOH, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, 0.5 mM PMSF, pH 7.9), left on ice for

20 min, vortexed, and centrifuged at 15,000 × *g* for 5 min at 4°C. Aliquots of the supernatant that contain nuclear proteins were frozen in liquid nitrogen and stored at -70°C. Protein was determined using a bicinchoninic acid (Sigma. St. Louis, MO, U.S.A.).

Statistical analysis

The results were expressed as mean ± S.E.M. for a number of experiments. Statistical significance was compared between each treated group and control by analysis of variance (ANOVA), with post hoc test of the means according to Tukey's method. For all tests, *P* value less than 0.05 was considered significant.

RESULTS

Samultang on HMC-1 viability

First of all, to test cytotoxic effect of Samultang, we performed MTT assay in HMC-1. Cells were treated with various concentrations of Samultang (0.01 - 1 mg/ml) for 30 min and then stimulated with PMA plus A23187for 24 h. As shown in Fig. 1, we show that Samultang does not significantly affect cell viability and have no toxicity on HMC-1.

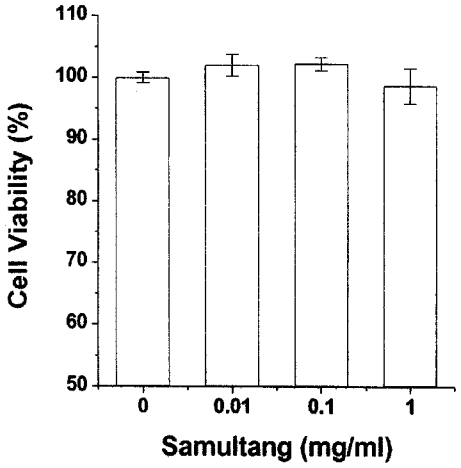

Fig. 1. Effect of Samultang on the cell viability in HMC-1. The cell viability was evaluated by MTT assay 24 h after Samultang treatment (0.01 - 1 mg/ml) in HMC-1. Data represent the mean ± S.E.M. of three independent experiments.

Effect of Samultang on cytokine production in HMC-1

We examined the inhibitory effect of Samultang on the PMA plus A23187-induced production of cytokine from HMC-1. Cells (5×10^5 cells/well) were pre-treated with Samultang (0.01 - 1 mg/ml) for 30 min and followed by cotreatment with PMA (50 nM) and A23187 (1 μM) for 8 h (for TNF-α and IL-6) or 24 h (for GM-CSF and VEGF). Then, the production level of cytokine was measured by ELISA method. Samultang inhibited production of TNF-α and IL-6. The inhibition rate of TNF-α production by Samultang (0.01 - 1 mg/ml) was about 34.36%, 70.73%, and 10.08%, respectively. The inhibition rate of IL-6 production by Samultang (0.01-1 mg/ml) was about 36.45%, 51.49%, and 17.11% respectively (Fig. 2A).

We also examined the inhibitory effect of Samultang on the PMA plus A23187-induced production of GM-CSF and VEGF from HMC-1. PMA plus A23187 significantly enhanced GM-CSF and VEGF production compared with media control. When Samultang was treated with Samultang (0.01 - 1 mg/ml), the inhibitory percentage to GM-CSF production by Samultang was about 39.94%, 54.03%, and 11.31% (Fig. 2B). The inhibitory percentage to VEGF production by Samultang was 46.45%, 47.95%, and 12.90%, respectively (Fig. 2C).

Effect of Samultang on COX-2 activation in HMC-1

To determine the effect of Samultang on COX-2 activation in PMA plus A23187-stimulated HMC-1, we performed the Western blot analysis. The cells were pre-treated with 0.1 mg/ml Samultang for 2 h and then treated with PMA plus A23187 for 12 h. As shown in Fig. 3, Samultang inhibited COX-2 expression levels induced by PMA plus A23187 (Fig. 3).

Effect of Samultang on NF-κB expression in HMC-1

NF-κB is a potent transcriptional activator of various inflammatory and immune mediator. In unstimulated cells, NF-κB is kept as inactive form

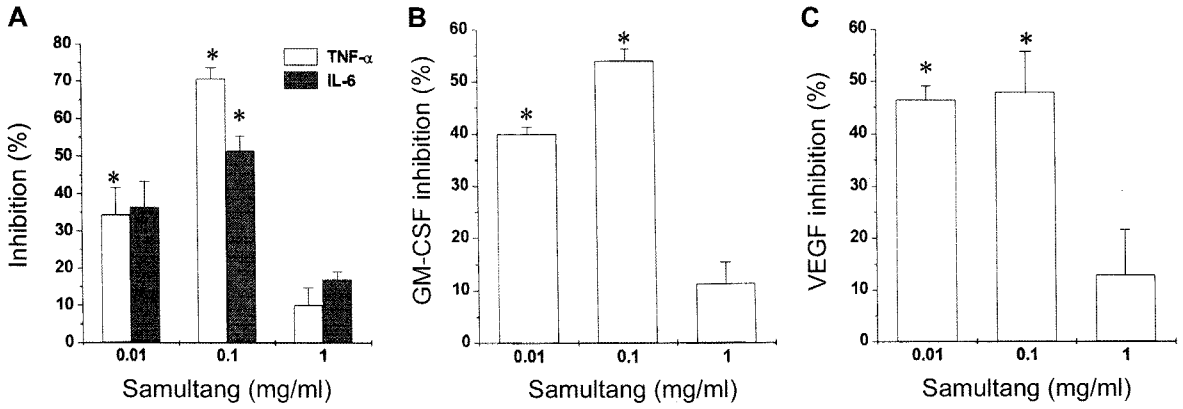

Fig. 2. Inhibition of cytokine production by Samultang in PMA plus A23187-stimulated HMC-1. The cells were pre-treated with Samultang for 30 min and then challenged with PMA plus A23187 for 8 h (for TNF-α and IL-6) or 24 h (for GM-CSF and VEGF). Samultang inhibited the production of TNF-α and IL-6 in PMA plus A23187-stimulated HMC-1 (A). Also, Samultang significantly inhibited the production of GM-CSF (B) and VEGF (C). The cytokine level was measured from cell supernatant using ELISA method. Values are mean ± S.E.M. of duplicate determinations from three separate experiments. $^{*}P < 0.05$; significantly different from the stimulated group.

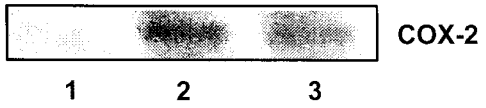

Fig. 3. Effect of Samultang on PMA plus A23187-Induced COX-2 activation in HMC-1 Cells. Cells were preincubated with 0.1 mg/ml Samultang for 2 h and then stimulated with PMA plus A23187 for 12 h. The protein level of COX-2 was determined by Western bolt analysis as described in Materials and Methods. 1, No stimulation; 2, PMA + A23187; 3, 0.1 mg/ml Samultang + PMA + A23187.

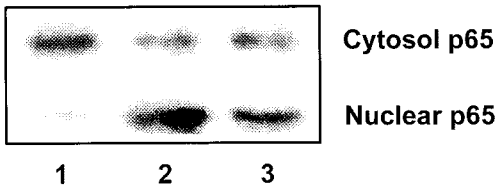

Fig. 4. Effect of Samultang on NF-kB activation. Cells were pre-incubated with 0.1 mg/ml Samultang for 2 h and then stimulated with PMA plus A23187 for 2 h. Cytoplasmic extracts and nuclear extracts were separated on 10% SDS-PAGE. After transferring onto nitrocellulase membrane, the membrane was immunoblotted with Abs for p65. 1, No stimulation; 2, PMA + A23187; 3, 0.1 mg/ml Samultang + PMA + A23187.

in the cytoplasm. After cell stimulation, NF-κB translocates into the nucleus. To investigate of NF-κB activation by Samultang, nuclear and cytoplasmic extracts were prepared and Western bolt analysis was performed with specific Ab NF-κB/*RelA* (p65 subunit). HMC-1 was pre-incubated with 0.1 mg/ml Samultang for 2 h and then challenged with PMA plus A23187 for 2 h. As shown in Fig. 4, we showed that PMA plus A23187 treatment considerably increased the nuclear *RelA*/p65 protein level and decreased the cytosolic *RelA*/p65, which is an indication of the nuclear translocation of RelA/p65. Pretreatment of Samultang inhibited the PMA plus A23187 stimulated increase and decrease of the nuclear and cytosolic *RelA*/p65 levels, respectively. These results suggested that Samultang blocks the nuclear translocation of the *RelA*/p65 from cytoplasm.

Effect of Samultang on MAPKs activation in HMC-1

To determined the effect of Samutang on MAPKs activation in PMA plus A23187-stimulated HMC-1, cells (2×10^6 cells/ml) were pre-treated with Samultang at 0.1 mg/ml for 30 min and followed by cotreatment with PMA plus A23187 for 15 min. Then total protein extracts were prepared and Western blot analysis was performed with specific Ab ERK, p38, and JNK. The pERK, pJNK, and pp38 levels were increased after PMA plus A23187

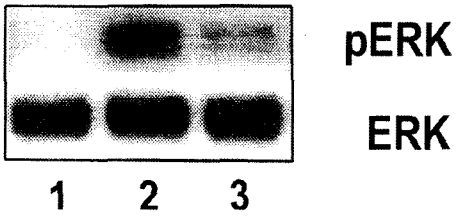

Fig. 5. Effect of Samultang on ERK activation. Cells were pre-incubated with 0.1 mg/ml Samultang for 30 min and then stimulated with PMA plus A23187 for 15 min. The protein level of phosphorylation of ERK and totoal ERK was determined by Western bolt analysis as described in Materials and Methods. 1, No stimulation; 2, PMA + A23187; 3, 0.1 mg/ml Samultang + PMA + A23187.

treatment. Fig. 5 shows that 0.1 mg/ml Samultang dramaticaly inhibited pERK activation (Fig. 5). However, Samultang did not inhibit pp38 and pJNK expression (data not shown).

DISCUSSION

The present study showed that Samultang pre-treatment inhibited the production of TNF-α, IL-6, GM-CSF, and VEGF in PMA plus A23187-stimulated mast cells. Samultang also inhibited PMA plus A23187-induced COX-2, NF-κB, and pERK activation. HMC-1 activated by PMA plus A23187 are useful *in vitro* model system for studying of multi-functional effects of the immune and inflammatory reactions (Nilsson *et al.*, 1995; Shin *et al.*, 2003). We showed that Samultang inhibited inflammatory cytokines such as TNF-α, IL-6, GM-CSF, and VEGF from PMA plus A23187-stimulated HMC-1.

The pro-inflammatory cytokine, TNF-α is produced and secreted under pathological conditions that are associated with increased pain and hyperalgesia, for example during neuropathies, tumor growth or in chronic inflammatory diseases like rheumatoid arthritis (Eastgate *et al.*, 1998; Watkins and Maier, 1999). IL-6 is thought to be one of the pro-inflammatory cytokines that plays important roles in the autoimmune response and inflammatory reaction. GM-CSF plays a pivotal role in inflammatory and immunologic processes (Gajewska, 2003). Release of GM-CSF in the airway can mediate acute inflammatory responses as well as initiate and perpetuate local immune responses. Elevated levels of GM-CSF have been well demonstrated in bronchoalveolar lavage fluid, endobronchial biopsy, and sputum samples from asthmatics (Soloperto *et al.*, 1991; Broide *et al.*, 1993; Oh *et al.*, 1999). VEGF is known to strongly promote angiogenesis and is found in increased levels at sites of chronic inflammation (Norrby, 2002; Abdel-Majid and Marshall, 2004). Recently, other research showed that human mast cells is a potent source of VEGF and it may provide new opportunities the regulate angiogenesis at mast cell rich-sites (Esposito *et al.*, 2004). In inflammation, infiltrating inflammatory cells and some resident cells are the producers of the VEGF (Freeman *et al.*, 1995; Appleton *et al.*, 1996). Current clinical approaches to the treatment of inflammation mainly focus on the inhibition of pro-inflammatory mediator production and the suppression of the initiation of the inflammatory response. Anti-TNF therapy has defined a molecular target and new approach for treating inflammatory disorders (Keystone, 2001; Taylor, 2001). Humanized anti-human IL-6 receptor monoclonal antibody has been clinically developed as a therapeutic agent for some autoimmune inflammatory diseases, such as rheumatoid arthritis and Crohn's disease, originally in Japan (Nishimoto, 2005). COX-2 is an inducible enzyme found at low concentration in healthy tissues, but it is up-regulated in response to tissue damage during inflammation. Several recent studies demonstrated that PGD2, which is the COX-2 metabolite released from activated mast cells, is also essential for the pathogenesis of eosinophilic airway inflammation. Previously, it has been reported that COX-2 inhibitors abolished PGD2 synthesis and attenuated eosinophil accumulation in the airways inflammation. In this study, we observed that Samultang inhibited the production of TNF-α, IL-6, GM-CSF, VEGF and expression of COX-2 induced- PMA plus A23187. These results suggested that Samultang has potential effect on

anti-inflammatory response through the regulation of inflammatory genes in mast cells.

It is documented that the NF-κB is involved in the induction of enzymes which mediated inflammatory processes such as COX-2 and inducible nitric oxide and involved in the induction of pro-inflammatory cytokines such as TNF-α, IL-6, GM-CSF, and VEGF. Since these pro-inflammatory molecules are regulated at the transcription level, NF-κB is a critical intracellular mediator of the inflammatory cascade. Therefore, we postulated that Samultang mediates its effects at least partly through suppression of NF-κB activation. In this study, we showed that the Samultang inhibited the NF-κB activation induced PMA plus A23187. From this, Samultang may modulate mast cell-mediated inflammation through inhibition of NF-κB activation.

MAPKs signalling pathways are known to regulate production and activity of inflammatory mediators through activation of transcription factors such as NF-κB, AP-1, and ATF in different target cells (Gupta *et al.*, 1996; Barchowsky *et al.*, 2000). Other study reported that ERK signaling pathway has been implicated in cytokine production in bronchial epithelial cells and mast cells as well as in chemokine release from murine fibroblasts. Taken together, we supposed that the regulation of ERK signaling pathway could modulate allergic inflammation. Therefore, we investigated whether Samultang affects the ERK pathway induced- PMA plus A23187 in HMC-1. Interestingly, Samultang strongly inhibited the pERK activation. However, Samultang did not affect the pp38 and pJNK activation. These results demonstrate that the anti-inflammation effect of Samultang, at least in part, might be derived through regulation of the ERK pathway.

In conclusion, Samultang had no cytotoxic effect and inhibited the production of TNF-α, IL-6, GM-CSF, and VEGF production and COX-2 expression in human mast cells. Moreover, Samultang inhibited NF-κB and ERK activation. These results may help in understanding the mechanism of action of Samultang leading to control activation of mast cells on inflammatory responses.

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