The Oriental Medicine ‘Cool-Cool (Cool-X-A)’ Inhibits Inflammatory Cytokine Production and Migration in Mast Cells

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Plant medications have been applied to treat pains from various types of arthritis in Korea. Rheumatoid arthritis (RA) is well known to be a chronic autoimmune/inflammatory disease that leads to progressive joint damage and cartilage destruction. Accumulation and activation of mast cells have been demonstrated in rheumatoid synovial tissue. Because infiltrated mast cells and their mediators may contribute to the initiation and progression of the inflammatory process and matrix degradation of RA, we tested the inhibitory effects of ‘Cool-Cool’ (CC, Cool-X-A), an Oriental medication, on the production and migration of major inflammatory cytokines in mast cells. CC was treated in vitro before activation of human mast cell line (HMC-1) with phorbol 12-myristate 13-acetate, and the cytotoxicity of CC was assessed by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide assay. CC had no cytotoxic effects on HMC-1 cell viability. The inhibitory effects on cytokine production were monitored by enzyme-linked immunosorbent assay and reverse transcriptase-polymerase chain reaction (RT-PCR). CC inhibited not only the secretion but also the expression of TNF-α and IL-8 in HMC-1 cells. CC also suppressed migration of mast cells induced by stem cell factor. These findings may help in understanding the mechanism of action of this herbal medication, leading to the control of mast cells in inflammatory conditions like RA.

Key words herbal medication; mast cell; tumor necrosis factor-α; interleukin-8; chemotaxis

‘Cool-Cool’ (CC, Cool-X-A) is a water extract made from six herbal medicines and the components are interesting from the viewpoint of an expectation of relieving rheumatoid arthritis (RA). This medication has been applied to treat pain from various types of arthritis in South Korea. However, it is still unclear how it regulates the immune or inflammatory responses.

Rheumatoid arthritis (RA) is one of the most typical rheumatic diseases, and is characterized by chronic inflammatory disease that leads to cartilage destruction, joint deformity, and disability.1,2) In the rheumatoid lesion, profound hypertrophic changes in the synovium with infiltration of immune cells, increased vascularity, and hyperplasia result in the formation of a synovial pannus that invades cartilage and bone.3) The cellular composition at sites of cartilage erosion varies greatly, such as macrophages, fibroblasts, mast cells, polymorphonuclear leukocytes, and dendrocytes.4)

Although mast cells have been viewed primarily in the central role of immediate-type hypersensitivity reactions,5,6) the significant contribution of mast cells in the pathogenesis of rheumatic diseases recently has become more evident. Accumulation of mast cells and their activation/degranulation have been demonstrated in rheumatoid synovial tissues as well as in the synovial fluids.7–9) Activated mast cells synthesize prostaglandins and leukotrienes, and release both preformed and newly synthesized cytokines such as tumor necrosis factor-α (TNF-α) and interleukins.10) Thus, infiltrated mast cells and their mediators may contribute to the initiation and progression of the distributive inflammatory process and matrix degradation of RA.11,12) Inhibition of mast cell accumulation or activation is becoming a new therapeutic approach to control chronic inflammation in the rheumatoid lesion.

The involvement of pro-inflammatory cytokines, particularly TNF-α resulting in local inflammation, in the pathogenesis of RA, is now well-accepted.13–15) TNF-α, perhaps the best studied, is an autocrine stimulator as well as a potent inducer of other inflammatory cytokines, including IL-1, IL-6, IL-8, and granulocyte-monoocyte colony-stimulating factor.16,17) TNF-α has been considered as a pivotal cytokine and a key mediator in the pathogenesis of RA and there is recent growing evidence for anti-TNF-α therapies.18,19) IL-8, the first human chemokine to be characterized, is often upregulated at inflammatory sites including rheumatoid lesions.20,21) This chemokine acts on inflammatory effector cells such as neutrophils, T-lymphocytes, B-lymphocytes and eosinophils inducing migration or release responses.22–25) Accordingly, the blockade of these cytokines is now the main target of new approaches to treatment.26–28)

Stem cell factor (SCF) is not only a mast cell chemotaxin but is also important for other mast cell functions, such as growth and differentiation, survival, adhesion, and activation.29,30) Recent reports show that synovial fibroblasts express SCF as well as induce mast cell chemotaxis, and mast cell chemotactic activity in synovial fluid.31,32) In the present study, we showed that CC inhibited the production of PMA-induced pro-inflammatory cytokines (TNF-α and IL-8) from human mast cells. We also examined the effects of CC on SCF-induced migration of mast cells.

MATERIALS AND METHODS

Reagents and Antibodies Cell culture medium, Iscove’s modified Dulbecco’s medium (IMDM) and RPMI 1640 were...
purchased from Gibco BRL (Grand Island, NY, U.S.A.). 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT), phorbol 12-myristate 13-acetate (PMA), avidin-HRP, 2,2-azio-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), PD98059 and other reagents were obtained from Sigma (St. Louis, MO, U.S.A.). For RT-PCR, commercially available primer sets of TNF-α and IL-8 were prepared from Stratagen (La Jolla, CA, U.S.A.). Anti-human TNF-α Ab, biotinylated anti-human TNF-α Ab, recombinant human TNF-α, and recombinant rat SCF were purchased from R&D Systems (Minneapolis, MN, U.S.A.). Anti-human IL-8 Ab, biotinylated anti-human IL-8 Ab, and recombinant human IL-8 were obtained from PharMingen (Cambridge, U.K.).

Preparation of CC Extract of CC was prepared by decoting the dried prescription of 6 herbs with 11 distilled water at 80°C. The extraction decoted for approximately 2 h (200 ml) was filtered, lyophilized, and kept at 4°C. The CC water extract powder was dissolved in PBS and filtered with a 0.2 μm syringe filter. CC contained 15 g of Rehmannia glutinosa LIBOCH, 15 g of Achyranthes bidentata, 15 g of Acanthopanax sessiliflorus SEEM, 5 g of Perilla frutescens L., 5 g of Atractylodes macrocephala KOIDZ, and 5 g Glycyrrhiza uralensis FISCH. All plants were obtained from Daehak Oriental Pharmacy (Iksan, South Korea).

Cell Culture and Stimulation Human leukemic mast cell line (HMC-1) was grown in IMDM supplemented with 100 unit/ml penicillin, 100 μg/ml streptomycin, 10−3 M monothioglycerol, and 10% heat-inactivated FBS at 37 °C in 5% CO2 and 95% humidity. HMC-1 cells (1×106 cells/ml) were treated with CC (1—100 μg/ml) for 30 min prior to stimulation with 50 nm PMA and incubated at 37 °C for additional time.

MTT Assay To test the viability of cells, MTT colorimetric assay was performed as described previously.33) Briefly, HMC-1 cells (5×104 cells/ml) were incubated for 8 h after stimulation with 50 nm PMA, in the absence or presence of CC (100 μg/ml). After addition of MTT solution, the cells were incubated at 37 °C for 4 h. The crystallized MTT was dissolved, and the absorbance was measured at 540 nm.

Assay of TNF-α and IL-8 Release Secreted cytokine levels in culture supernatants of HMC-1 were measured by a sandwich enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s protocol (for TNF-α assay, R&D Systems; for IL-8 assay, PharMingen). Absorption of the avidin-horseradish peroxidase color reaction was measured at 405 nm and compared with serial dilutions of human recombinants as a standard.

RT-PCR The total RNA was extracted from the harvest cells using a high pure RNA isolation kit (Roche Molecular Biochemicals, Germany) and 1 μg of total RNA was converted to cDNA by reverse transcriptase at 37°C for 1 h using a first-strand cDNA synthesis kit (Amersham Pharmacia Biotech, Germany). The PCR amplification consisted of 35 cycles (94°C, 45 s; 60°C, 45 s; 72°C, 1.5 min) with the commercial oligonucleotide primer sets for human TNF-α, IL-8, and β-actin. These primer sets yield PCR products of 355, 200, and 661 bp for TNF-α, IL-8, and β-actin, respectively. The final PCR products were electrophoresed on 2% agarose gels.

Chemotaxis Assay To test migration of the mast cells, we isolated peritoneal mast cells from rats (RPMCs). The isolation method was previously described.34) SCF (10 ng/ml) used for inducing chemotaxis was placed in the lower chambers of the wells (Nalge Nunc International, Roskilde, Denmark). After 10-mm migration filter inserts (Nalge Nunc International) were placed into each well, 5×104 RPMCs were added into each insert. The lower compartment of the well was separated from the cell suspension in the upper compartment with an 8 μm pore-size polycarbonate membrane of the culture inserts. After incubation with CC (1 μg/ml) for 1 h, RPMCs were additionally incubated for 4 h at 37°C in a humidified atmosphere flushed with 5% CO2 in air. Following aspiration of nonadherent RPMCs in the upper compartment, cells adherent to the upper surface of the membrane were removed by scraping with a rubber blade. Migrated cells adherent to the lower surface of the membrane were fixed with methanol for 5 min and stained with 0.5% toluidine blue. The membranes were mounted on glass slides by routine histological methods. The number of mast cells that migrated across the membrane was counted and averaged under a light microscope.35) Migration numbers on each filter were calculated as the mean of the readings of three different areas of the filter.

Statistical Analysis Results are expressed as the mean±S.E.M. of the indicated number of experiments. The significance of the differences between the PMA-treated groups and the control group, and between the PMA-treated groups and the CC-treated groups, were determined by the Mann–Whitney U-test. For all tests, p values less than 0.05 were considered significant.

RESULTS

Cytotoxic Effects of CC on HMC-1 Viability To test the cytotoxic effect of CC, we performed MTT assay in HMC-1 cells. Figure 1 shows the viability of cells incubated for 8 h after stimulation with PMA (50 nm) in the absence or presence CC (1—100 μg/ml). In the cells treated with PMA, cell viability decreased slightly to 90.1±13.2% compared with the control value of 100±2.7%. However, CC did not affect cell viability in any condition and was not toxic to HMC-1 cells.

Inhibitory Effects of CC on TNF-α and IL-8 Secretion HMC-1 cells can be induced by non-physiological agents such as PMA or A23187 to release the various cytokines. In preliminary experiments, we determined the optimal time for...
secretion of TNF-α and IL-8 by PMA (data not shown). TNF-α secretion reached a peak at 8 h of incubation after PMA stimulation. An approximate twenty-fold increase in TNF-α was obtained in the PMA-treated supernatant after 8 h compared with no stimulation (Fig. 2A). The inhibitory effects of CC on TNF-α secretion were examined in PMA-induced HMC-1 cells. CC pretreatment in cells inhibited PMA-induced TNF-α secretion and 1 μg/ml CC treatment blocked TNF-α secretion by 52.5±11.1% compared with no CC treatment (p<0.05). As shown in Fig. 2B, the effects of CC on IL-8 secretion were evaluated. The amount of IL-8 released into the supernatants after 8 h stimulation increased almost thirty-fold compared with no stimulation. CC inhibited IL-8 secretion from the cells, and only 1 μg/ml CC exhibited statistical significance (by 50.7±11.4% inhibition).

Effects of CC on TNF-α and IL-8 mRNA Expression

In order to determine whether CC can regulate cytokine mRNA expression, total RNA was isolated from HMC-1 cells and reverse-transcribed into cDNA. The cDNA was amplified by PCR with primers specific for TNF-α and IL-8 and the final PCR products correspondingly yielded 355 and 200 bp bands on 2% agarose gels (Fig. 3). PCR using housekeeping gene human β-actin (661 bp) was carried out to confirm the equivalency of the cDNA preparations. After incubation in PMA for 7 h, TNF-α expression was increased compared with the absence of PMA, and CC suppressed the TNF-α expression. IL-8 expression was also increased compared with the absence of PMA. CC treatment reduced the PMA-induced IL-8 expression, however, CC failed to dramatically suppress IL-8 expression in HMC-1 cells.

Inhibitory Effects of CC on Migration of Mast Cells

SCF (50 ng/ml) was placed in the lower compartment, and then RPMCs were incubated for 4 h in the upper compartment. The number of RPMCs that migrated toward the lower surface of the polycarbonate membrane through 8 μm pores were markedly increased 2 and 4 h later. The maximum number reached was 158±16 cells. As shown in Fig. 4, the addition of SCF resulted in a significant increase in the number of migrated RPMCs. Pretreatment with CC inhibited the SCF-induced chemotactic response by 64.2±7.9% (by 61±12 cells). In the case of migration by SCF, RPMCs were still migrating 4 h later and cells that were detached from the membrane toward the lower compartment were detected. In contrast, medium alone and CC alone without SCF had no effects on the migration of RPMCs in cultures up to 4 h.

DISCUSSION

In this study, we showed that CC regulated the production of pro-inflammatory cytokines in mast cells and the migration of mast cells. Human leukaemic mast cell line HMC-1 exhibits mast cell characteristics and is a useful in vitro model system for studying the multifunctional effects of immune and inflammatory reactions. We also observed an activation of HMC-1 cells by PMA- or A23187-stimulation and an induction of pro-inflammatory cytokines in the cells.

TNF-α reportedly plays a pivotal role in the pathogenesis of mast cell activation and anaphylaxis. This study showed that CC suppressed cytokine expression and migration of mast cells induced by SCF. These findings suggest that CC may be a new therapeutic approach for the treatment of inflammatory diseases.
of RA, especially cartilage and bone degradation, and this cytokine is highly up-regulated in RA. TNF-α is able to regulate IL-1β expression, which is important for the induction of prostanoid and matrix metalloproteinase production by synovial fibroblasts and condrocytes. Thus, anti-TNF therapy for RA has defined a molecular target and is a new approach for treating immunoo-inflammatory disorders. Although TNF-α expression by monocytes/macrophages has been mainly demonstrated both in synovial tissue and at the cartilage-pannus junction, it is believed that mast cells also have numerous functional roles, including the regulation of TNF-α in RA. In RA, the immune and inflammatory responses are amplified by stimulating T cells, mononuclear cells, synovial fibroblasts, and synovial mast cells. The human chemokine, IL-8, released from mast cells might act on surrounding cells such as neutrophils, T- and B-lymphocytes, and eosinophils, and induce migration and activation of inflammatory effector cells.22—25) The release of pro-inflammatory cytokines causes synovial inflammation and joint damage as a result of the degradation of connective tissue. Clearly, there are many possible therapeutic targets, but the regulation of pro- and anti-inflammatory cytokines would seem to offer an especially useful approach to suppressing inflammation and preventing joint damage.27,37)

SCF is not only a mast cell chemotaxin but is also important for other mast cell functions, such as growth and differentiation, survival, adhesion, and activation. Indeed, synovial mast cells express functional SCF receptors (c-kit/CD117).38,39) The expression of SCF was promoted by TNF-α, a key regulator in synovitis. These interactions play an important role in mast cell accumulation and related events in RA. In the present study, CC effectively inhibited mast cell migration induced by SCF in an in vitro chemotactic assay.

In conclusion, CC had no cytotoxic effects and inhibited the production of TNF-α and IL-8 in mast cells. CC inhibited the mRNA expression of TNF-α, but did not inhibit the expression of IL-8. In addition, CC suppressed migration of mast cells induced by SCF. These findings may be important in understanding the action of CC leading to control mast cells. In order to elucidate the mechanism of action of CC, we investigated the effect of CC on the activation of mitogen-activated protein kinases. They were activated by PMA stimulation in HMC-1, but CC did not affect the activation (data not shown). Therefore, this point still needs to be studied further.

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