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Anti-Allergic Effect of *Ponciri fructus*

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The immature fruits of *Poncirus trifoliata* L. or *Ponciri fructus* (PF), well known as 'Jisil' in Korea, have been used against allergic diseases for generations, and still occupy an important place in traditional Oriental medicine. Anti-allergic effects of this fruit have been investigated in a few experimental models.

Immunoglobulin E (IgE) is the principal immunoglobulin involved in immediate hypersensitivities and chronic allergic diseases. The effect of an aqueous extract of PF on *in vivo* and *in vitro* IgE production was investigated. PF dose-dependently inhibited the active systemic anaphylaxis and serum IgE production induced by immunization with ovalbumin, *Bordetella pertussis* toxin and aluminum hydroxide gel. PF strongly inhibited interleukin 4 (IL-4)-dependent IgE production by lipopolysaccharide-stimulated murine whole spleen cells. In the case of U266 human IgE-bearing B cells, *Ponciri fructus* also showed an inhibitory effect on the IgE production.

On the other hand, mast cell hyperplasia can be causally related with chronic inflammation. Stem cell factor (SCF), the ligand of the c-kit protooncogene product, is a major regulator and chemoattractant of mast cells. Ponciri fructus (1 mg/mL) significantly inhibited the SCF-induced migration of rat peritoneal mast cells (RPMCs). RPMCs exposed to SCF (50 ng/mL) resulted in a drastic shape change with a polarized morphology while the cells exposed to *Ponciri fructus* (1 mg/mL) remained resting, with little or no shape alteration. The drastic morphological alteration and distribution of polymerized actin were blocked by pretreatment with *Ponciri fructus*. In addition, *Ponciri fructus* inhibited both TNF-alpha and IL-6 secretion from RPMCs stimulated with SCF.

These results suggest that *Ponciri fructus* has an anti-allergic activity by inhibition of IgE production from B cells. These findings also provide evidence that *Ponciri fructus* inhibits chemotactic response and inflammatory cytokines secretion to SCF in mast cells.

INTRODUCTION

Allergic diseases such as asthma, allergic rhinitis, atopic dermatitis and food allergy afflict up to 20% of the human population in most countries and are believed to be increasing in prevalence (Wuthrich, 1989). The etiology of allergic reactivity and its basis in immunoglobulin E (IgE)-mediated pharmacological processes of a variety of cell populations such as mast cells and eosinophils is well recognized (Stevens and Austen, 1989). It has been recognized that antibody response in animals is greatly influenced by the adjuvant used for immunization. For example, *Bordetella pertussis* vaccine is known to be potent adjuvant for production of IgE antibody in the rat and mouse (Itaya et al., 1980). It has been demonstrated that aluminum hydroxide gel (alum) is extremely effective in eliciting IgE antibody in certain animal species (Revoltella and Ovary, 1969). The critical role of IL-4 in regulating IgE production is also now established, as are the sources of IL-4 in Th2-type T cells and mast cells (Mosmann et al., 1986; Snapper et al., 1988; Hikida et al., 1996).

Mast cells are multifunctional effector cells of the immune system (Galli, 1990). These cells produce and store large amounts of proinflammatory mediator substances, including histamine, prostaglandins, proteolytic enzymes,

tumor necrosis factor (TNF)-α, and other cytokines (Serafin and Austen, 1987). In response to diverse stimuli, mast cells release these mediators to the extracellular space (Ishizaka and Ishzaka, 1984). During inflammatory reactions, mast cells can be activated by a specific antigen(s), by complement degradation products, or by other immune mediators. A primary growth factor involved in mast cells function has been identified as the *c-kit* ligand or stem cell factor (SCF) (Tsai et al., 1991). SCF, the ligand of the *c-kit* protooncogene product, is suggested to be a major regulator and chemoattractant of mast cells. In particular, SCF induces differentiation of mast cells from their progenitor cells (Irani et al., 1992). SCF is a well-recognized regulator of mature mast cells and modulates their secretory capacity as well as directed migration (chemotaxis). In addition, numerous mast cells accumulate at local tissues for various conditions such as wound healing, tumors, host defence responses against helminth parasites and ectoparasites, and acute and chronic allergic disorder (Nilsson et al., 1994).

The immature fruits of *Poncirus trifoliata* L. (*Rutaceae*) or *Poncirus fructus* (PF), well known as 'Jisil' in Korea, have been used against allergic diseases for generations, and still occupies an important place in traditional Oriental medicine. It is previously reported that PF inhibited mast cell-mediated anaphylactic reactions (Lee et al., 1996). We have investigated the effect of PF on IgE production and on the SCF-induced migration, morphological alterations, and cytokines (TNF-α and IL-6) secretion in primary cultures of RPMCs.

RESULTS

Groups of mice ($n = 10$ group) were orally treated with saline or PF given at various doses for 2 weeks. Antigen mixture was intraperitoneally given to the group of mice. Mortality (%) within 1 h following ovalbumin challenge is presented as [(number of dead mice)/(total number of experimental mice)] $\times$ 100. PF dose dependently inhibited antigen mixture-induced active systemic anaphylaxis. The dose giving 50% inhibition (ID_{50}) was about 200 mg/kg for PF. PF showed the maximum inhibition at dose of 400 mg/kg.

The ability of PF to influence antigen mixture- induced serum IgE production was investigated. Groups of mice ($n = 10$ group) were orally treated with saline or PF given at various doses for 2 weeks. Antigen mixture was intraperitoneally given to the group of mice. PF dose-dependently inhibited antigen mixture-induced IgE production at doses of 50～800 mg/kg. PF showed maximum inhibition at a concentration of 400 mg/kg.

IL-4 can induce IgE class switching in mouse and human activated B cells (Coffman et al., 1993). In the supernatants of LPS-stimulated whole spleen cells in the presence of IL-4 and PF, IgE was assayed on day 7. PF strikingly inhibited the LPS-dependent or LPS plus IL-4-dependent IgE production by whole spleen cells.

Culture of spleen cells for 7 days in the presence of LPS increased the number of cells. In the presence of LPS, PF at the concentration of 1000 µg/mL did not affect the cell number.

The influence of PF addition on IgE production by LPS-stimulated or LPS plus IL-4-stimulated U266B1 cells was studied. PF inhibited the LPS-dependent or LPS plus IL-4-dependent IgE production by U266B1 cells. In U266B1 cells, IgE productions were detected as high levels; the IgE levels were from 193.4 $\pm$ 10.0 to 382.3 $\pm$ 37.5 ng/mL. No significant cytotoxicity of PF on culture was observed in the concentrations used in the experiments as assessed by Trypan blue uptake. In addition, PF also did not significant influence the proliferation of the U266B1 cells.

To see the effect of PF on migration of RPMCs SCF (50 ng/mL) was placed in the lower compartment, and then RPMCs were incubated for 4 h in upper compartment. RPMCs migrated toward the lower surface of the polycarbonate membrane through 8 µm pores were markedly increased 4 h later. The addition of rmSCF resulted in a significant increase in the number of migrated RPMCs, but pretreatment with PF (1 mg/mL) inhibited the

rmSCF-induced chemotactic response by 72.9%. The migration of RPMCs was not inhibited at the lower concentrations (0.001～0.1 mg/mL) of PF. We next observed Toluidin blue positive mast cells on the membrane by treatment with rmSCF, but we nearly made no observation in culture for up to 4 h by pretreatment with PF.

After stimulation with rmSCF (50 ng/mL) for 4 days, we experimented whether PF can block shape change of RPMCs on the polycarbonate membrane fixed with methanol for 5 min. The surface structure of RPMCs migrating on the lower surface of the membrane through pores was scanned with an atomic force microscope. RPMCs exposed to rmSCF (50 ng/mL) resulted in a drastic shape change with a polarized morphology, while RPMCs exposed to pretreatment with PF (1 mg/mL) remained resting, with little or no shape alteration.

As F-actin formation is well known to be associated with cell motility, we next examined migratory RPMCs on the distribution of F-actin taken from RPMCs stimulated by rmSCF (50 ng/mL) using confocal laser scanning microscopic analysis. The cells were stained with Oregon Green 488-phalloidin, and a strong positive reaction was detected. To determine whether the polymerization of actin filaments was inhibited by PF, RPMCs were pretreated with PF (1 mg/mL) for 1 h. We observed that PF inhibited the rmSCF-mediated F-actin formation.

Finally, to assess the effect of PF in rmSCF-induced both TNF-α and IL-6 secretion, RPMCs were pretreated with PF for 1 h prior to rmSCF treatment. The inhibitory effect of PF on rmSCF-induced TNF-α secretion was significant ($p<0.005$) at doses of 0.1, 1 mg/mL. PF (1 mg/mL) significantly ($p<0.001$) inhibited rmSCF-induced IL-6 secretion. IL-6 secretion was not inhibited at the lower concentrations (0.001～0.1 mg/mL) of PF. Mast cell viability has not been affected at 1 mg/mL concentration of PF.

DISCUSSION AND CONCLUSIONS

The present study demonstrates that PF inhibits IgE production *in vivo* and *in vitro*, and also shows that PF inhibits IgE production of human B cell line. Indeed, PF at the doses of 0.1 and 1 mg/mL suppressed the induction of IgE production by non-stimulated whole spleen cells. This suppression is not due to the interference with the assay for the detection of IgE, because the value of PF-treated group increased rather than the saline group in the U266B1 cells. PF inhibited T celldriven IL-4-dependent IgE synthesis by whole spleen cells and U266B1 cells. Inhibition of IgE production was not due to toxicity to either whole spleen cells or U266B1 cells. Neither the proliferation nor the viability of whole spleen cells or U266B1 cells was affected even at the highest concentrations of PF (1 mg/mL) we used.

Interestingly, PF not only cancelled the stimulatory effect of LPS or LPS plus IL-4 on the IgE production, but also decreased the production to the level lower than the saline level in whole spleen cells. However, PF did not affect IgE production by non-stimulated U266B1 cells. PF may specifically contribute to the inhibition of IgE production from whole spleen cells and purified B cells. To clarify the IgE production regulating mechanism of PF, further studies are needed. IL-4 can induce IgG1 (IgG4) and IgE class switching in mouse and human activated B cells (Coffman et al., 1993). Active systemic anaphylaxis may involve both IgG and IgE class antibody. The effects of IL-4 on the stimulation of IgG secretion in vitro are somewhat more complex. IL-4 stimulates IgG1 secretion and inhibits the secretion of other isotypes of IgG by LPS blasts cultured in the absence of T lymphocytes (Vitetta et al., 1985; Snapper et al., 1988). In addition, anti-IL-4 antibody inhibits IgG1 secretion by B cells cultured with anti-IgM antibodies plus a culture supernatant produced by activated T cells (Isakson, 1986). In contrast, anti-IL-4 antibody has only a modest suppressive effect on the IgG1 response made by B cells cultured with rabbit anti-IgM antibody and rabbit IgG-specific Th cells (Coffman et al., 1988), and IL-4 actually suppresses antigen-specific IgG responses, including those of the IgG1 isotype, that are made by antigen-primed B cells in

the presence of high concentrations of antigen and antigen-primed B cells in the presence of high concentrations of antigen and antigen-specific T cell lines (Asano et al., 1988). Furthermore, even in *in vitro* systems in which IL-4 is an important stimulator of IgG1 secretion, the concentration of IL-4 that is required to stimulate maximal IgG1 secretion is approximately 100- fold less than that required to stimulate maximal secretion of IgE; and higher IL-4 concentrations that more effectively stimulate IgE secretion, especially if maintained for more than 2 days, can inhibit IgG1 secretion (Snapper et al., 1988). These complexities made it difficult to predict what effects IL-4 would have on IgG antibody responses *in vivo*.

It was found that a hypothalamic pituitaryadrenal axis of the endocrine system plays an important role in the regulation of Th1:Th2 balance (Rook et al., 1994). The effect of PF in vivo may decrease Th2 activity and IL-4 production by Th2 cells. Thus, it may be concluded from the results of the present study that PF possess anti-allergic activity by inhibition of Th2 activity.

We demonstrated that PF prevents rmSCF-induced migration, shape alteration, and formation of F-actin of RPMCs. In addition, PF inhibits the secretion of TNF-α and IL-6 in SCF stimulated RPMCs. These results indicate that PF inhibits the further development of RPMCs in response to rmSCF.

SCF is a major regulator of mast cells, promoting survival, differentiation, adhesion, chemotaxis, and mediator secretion (Galli, 1994). In addition, soluble SCF reportedly induces mast cells chemotaxis (Nilsson et al., 1994). It was tempting to speculate that SCF is involved in the accumulation of mast cells in various allergic diseases. Rheumatoid arthritis is a chronic inflammatory disease characterized by mast cell hyperplasia. Recently mast cells have been recognized as an important effector cell of the rheumatoid lesion (Marone, 1998). Directed migration of a variety of inflammatory cells toward a chemical gradient of specific chemoattractants locally produced in inflamed tissues is the first integrated event in the process of allergic and nonallergic inflammatory responses (Baggiolini, 1998).

Chemoattractant ligands stimulate specific receptors on the cell surface that initiate several second messenger cascades; this action results in a change in F-actin distribution from azimuthal symmetry around the cell rim to concentration at a particular region involved in migratory behavior (Coates, 1992). In this study, we found that PF inhibited the SCF-induced migration, shape change, and F-actin formation of RPMCs. In view of the present results, the allergy and rheumatoid arthritis pathologies potentially related to inflammatory reactions associated with mast cell hyperplasia are expected to be sensitive to PF treatments. We need further study to elucidate whether PF affects the binding of SCF to mast cells using a labeled ligand, in order to explain the inhibitory mechanism of PF.

The secretion of TNF-α has been demonstrated *in vitro* and *in vivo* studies in mice. Human skin mast cells also point to the early secretion of preformed TNF-α (Walsh et al., 1992). The results of the present study showed the ability of RPMCs to secrete small amounts of TNF-α. In our experiments, we found that PF was able to inhibit TNF-α secretion by SCF-untreated control level. PF also inhibited IL-6 secretion from SCF-treated RPMCs. The results obtained in the present study provide evidence that PF may contribute to the prevention or treatment of various inflammatory diseases because these cytokines play an important role in initiating inflammation. These results suggest that PF may contain compounds with actions that inhibit mast cell migration. Further work should address the possibility that such component of PF may also be active in the inhibition of human mast cells. In addition, this effect may explain additional mechanisms for the therapeutic action of PF, particularly in chronic inflammation.

REFERENCES

Asano Y, Nakayama T, Kubo M, Fujisowa I, Karasuryama H, Singer A, Hodes RJ, Tada T. 1988. Analysis to two distinct B cell activation pathways mediated by a monoclonal T helper cell. II. T helper cell secretion of interleukin 4 selectively inhibits antigen-specific B cell activation by cognate, but not noncognate, interactions with T cells. *Journal of Immunology* 140: 419-426.

Baggiolini M. 1998. Chemokines and leukocyte traffic. *Nature* 392: 565-568.

Coates TD, Watts RG, Hartman R, Howard TH. 1992. Relationship of F-actin distribution to development of polar shape in human polymorphonuclear leukocytes. *J Cell Biol* 117: 765-774.

Coffman RL, Lebman DA, Rothman P. 1993. Mechanism and regulation of immunoglobulin isotype switching. *Advances in Immunology* 54: 229-270.

Coffman RL, Seymour B, Lebman DA, Kiraki DD, Christiansen JA, Shrader B, Cherwinski H, Savelkoul HFJ, Finkelman FD, Bond MW, Mosmann TR. 1988. The role of helper T cell products in B cell differentiation and isotype regulation. *Immunological Reviews* 102: 5-28.

Dimitriadou V, Koutsilieris M. 1997. Mast cell-tumor cell interactions: for or against tumor growth and metastasis. *Anticancer Res* 17: 1541-1549.

Galli SJ, Zsebo KM, Geissler EN. 1994. The kit ligand, stem cell factor. *Adz Immunol* 55: 1-96.

Galli SJ. 1990. New insights into the riddle of the mast cells: micro-environmental regulation of mast cell development and phenotypic heterogeneity. *Lab Invest* 62: 5-33.

Hikida M, Takai T, Ohmori H. 1996. Requirements of a costimulus for IL-4 induced IgE class switching in murine B cells activated via antigen receptors. *Journal of Immunology* 156: 2730-2736.

Irani AM, Nilsson G, Miettinen U, Craig SS, Ashman LK, Ishihzaka T, et al. 1992. Recombinant human stem cell factor stimulates differentiation of mast cells from dispersed human fetal liver cells. *Blood* 80: 3009-3021.

Isakson P. 1986. Anti-immunoglobulin-treated B cells respond to a B cell differentiation factor for IgG1. *Journal of Experimental Medicine* 164: 303-308.

Ishizaka T, Ishzaka K. 1984 Activation of mast cells for mediator release through IgE receptors. *Prog Allergy* 34: 188-235.

Itaya T, Tomioka H, Kumagai A, Tada T. 1980. Suppression of IgE antibody formation in mice with nonspecific stimulation. *Int Arch Allergy Appl Immunol* 62: 389-396.

Lee YM, Kim DK, Kim SH, Shin TY, Kim HM. 1996. Antianaphylactic activity of *Poncirus trifoliata* fruit extract. *J Ethnopharm* 54: 77-84.

Marone G. 1998. Mast cells in rheumatic disorders: mastermind or workhorse. *Clin Exp Rheumatol* 16: 245-249.

Mosmann TR, Cherwinski H, Bond MW, Geidlin MA, Coffman RL. 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *Journal of Immunology* 136: 2348-2357.

Nilsson G, Butterfield JH, Nilsson K, Siegbahn A. 1994. Stem cell factor is a chemotactic factor for human mast cells. *J Immunol* 153: 3717-3723.

Revoltella R, Ovary I. 1969. Reaginic antibody production in different mouse strains. *Immunology* 17: 45-54.

Rook GA, Hernandez-Pando R, Lightman SL. 1994. Hormones, peripherally activated prohormones and regulation of the Th1:Th2 balance. *Immunology Today* 15: 301-303.

Serafin I, Austen KF. 1987. Mediators of immediate hypersensitivity reactions. *N Engl J Med* 317: 30-34.

Snapper CM, Finkelman FD, Paul WE. 1988. Differential regulation of IgG1 and IgE synthesis by interleukin 4. *Journal of Experimental Medicine* 167: 183-196.

Stevens RL, Austen KF. 1989. Recent advances in the cellular and molecular biology of mast cells. *Immunology Today* 10: 381-386.

Tsai M, Shih L, Newlands GFJ, Takeishi T, Langley KE, Zsebo KM, et al. 1991. The rat c-kit ligand, stem cell factor, induces the development of connective tissue-type and mucosal mast cells *in vivo*. Analysis by anatomical

distribution, histochemistry and protease phenotype. *J Exp Med* 174: 125-131.

Vitetta ES, Ohara J, Myers C, Layton J, Krammer PH, Paul WE. 1985. Serological, biochemical, and functional identity of B cell-stimulatory factor 1 and B cell differentiation factor for IgG1. *Journal of Experimental Medicine* 162: 1726-1731.

Walsh LJ, Treinchieri G, Waldorf HA, Whitaker D, Murphy GF. 1991. Human endothelial leukocyte adhesion molecule 1. *Proc Natl Acad Sci USA* 88: 4220-4224.

Wuthrich B. 1989. Epidemiology of the allergic diseases: are they really on the increase? *Int Arch Allergy Appl Immunol* 90 (Suppl 1): 3-10.