Astilbin inhibits contact hypersensitivity through negative cytokine regulation distinct from cyclosporin A

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Background: IL-10 is known as a negative regulator for inflammatory diseases, including contact dermatitis. However, only a few drug candidates are reported to induce endogenous IL-10.

Objective: We sought to elucidate a new mechanism underlying the immunosuppressive properties of astilbin through negative cytokine regulation in comparison with the effective pattern with cyclosporin A.

Methods: Contact hypersensitivity was induced in mice with picryl chloride. Lymph node cells were isolated for adoptive transfer and cytokine assays.

Results: Astilbin significantly inhibited contact hypersensitivity when given in the elicitation phase but not in the sensitization phase, whereas cyclosporin A inhibited both phases. Lymph node cells from donor mice administered astilbin failed to adoptively transfer the hypersensitivity. Astilbin *in vivo* remarkably induced IL-10 expression in lymph node cells at an earlier time and decreased TNF-α and IFN-γ expression at a later time. Furthermore, the *in vivo* neutralization of IL-10 significantly impaired the effect of astilbin on contact hypersensitivity. In the isolated lymphocytes sensitized with picryl chloride *in vivo* and challenged with trinitrobenzene–sulfonic acid *in vitro*, astilbin did not affect the cell proliferation but modulated the above cytokine profiles as its *in vivo* effect in a concentration-dependent manner and furthermore significantly enhanced the expressions of suppressor of cytokine signaling 1 and 3. On the other hand, cyclosporin A strongly inhibited proinflammatory cytokine production but influenced neither IL-10 nor downstream suppressor of cytokine signaling 1 and 3 expression.

Conclusion: Astilbin alleviates contact hypersensitivity through a unique mechanism involving a negative cytokine regulation through stimulating IL-10, which is distinct from the immunosuppressant cyclosporin A. (J Allergy Clin Immunol 2005;116:1350-6.)

Key words: *Astilbin, immunosuppressant, contact hypersensitivity, picryl chloride, IL-10, TNF-α, IFN-γ*

Abbreviations used

DTH: Delayed-type hypersensitivity
MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
SI: Stimulation index
SOCS: Suppressor of cytokine signaling
SRBC: Sheep red blood cell
TNBS: 2,4,6-Trinitrobenzenesulfonic acid hydrate

Contact hypersensitivity as an animal model of T cell–mediated allergic contact dermatitis has been studied extensively. The pathogenesis of this disease can be characterized as delayed-type hypersensitivity (DTH) and separated into 2 phases. In the sensitization phase the antigen-presenting cells present antigenic peptides to naive T cells to make them activate and proliferate. In the elicitation phase the hapten-specific T cells are activated by the re-exposure of the same hapten to the skin and recruited at the site of contact. Cytokines and chemokines produced by the skin cells and the effector T cells significantly contribute to this process.[1] Among them, the T_H1-like cytokines, including TNF-α and IFN-γ, are predominantly involved in the response as proinflammatory factors. On the other hand, this DTH reaction is self-limited, and IL-10 is considered a natural suppressant of cutaneous inflammatory response.[2] Recent studies have shown that the anti-inflammatory function of IL-10 involves the induction of the suppressor of cytokine signaling (SOCS) family, especially SOCS-1 and SOCS-3.[3] These findings suggest that the modulation for IL-10 might be used as a new strategy for the treatment of some inflammatory diseases. For this purpose, it might be more important to find a drug that induces endogenous IL-10 production rather than to use an exogenous IL-10.

In addition to allergic contact dermatitis, many other immune-related conditions, such as multiple sclerosis, rheumatoid arthritis, and transplantation, have been known to involve the DTH mechanism.[4] However, there is still a lack of ideal therapeutic approaches to cure these diseases, except for the use of immunosuppressants, which show a strong anti-DTH activity.[5] These agents, such as glucocorticoids, cyclophosphamide, and even cyclosporin A, usually have severe side effects because of their nonselective targeting to the process of immune response and to the cell populations involved. To find a new immunosuppressant with low toxicity, our previous

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studies have confirmed the selective immunosuppressive activity of some kinds of Chinese herbs against a stage of DTH reaction.[6-8] As a special compound, astilbin, a flavanone isolated from Rhizoma Smilacis Glabrae, showed a selective immunosuppressive feature when administered orally during the elicitation, but not the sensitization, phase of various DTH reactions.[9] Before our findings, there have been only a few reports about this compound on its effects other than immunosuppression, such as antioxidative,[10] insecticidal,[11] antinociceptive, and antiedematogenic[12] activities. We further demonstrated that astilbin also improved immunologic liver injury with a DTH mechanism,[13] concanavalin A–induced liver injury,[14] and collagen-induced arthritis.[15] Its mechanism was found to involve a significant induction of apoptosis in the liver-infiltrating T lymphocytes[13] and mitogen-activated Jurkat T cells[16] and a selective suppression of activated T-cell adhesion[14] and migration.[9,15] These findings suggest that astilbin has a unique immunosuppressive pattern, selective inhibition on activated T cells, which is different from the present immunosuppressants. Such effectiveness might be advantageous for the treatment of immune diseases. Therefore the purpose of the present study is to explore its possible mechanisms and distinct advantages from the aspect of cytokine regulation through comparison with the immunosuppressant cyclosporin A, which has been widely used for the prevention of allograft rejection and for the treatment of various autoimmune diseases, including dermatitis.[17]

METHODS

Animals

Female ICR mice (5-6 weeks old, 18-22 g) were supplied by the Experimental Animal Center of Nanjing Medical University (Nanjing, China), and female BALB/c mice (6-8 weeks old, 18-22 g) were supplied by the Laboratory Animal Center of Shanghai (Shanghai, China). They were maintained with free access to pellet food and water in plastic cages at 21°C ± 2°C and kept on a 12-hour light-dark cycle. Animal welfare and experimental procedures were carried out strictly in accordance with the "Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) and the related ethical regulations of our university. All efforts were made to minimize the animals' suffering and to reduce the number of animals used.

Drugs and reagents

Astilbin, 3,3′,4′,5,7-pentahydroxyflavanone 3-(6-deoxy-[L-mannopyranoside]), was isolated from the rhizome of *Smilax glabra*, a Liliaceae plant. Cyclosporin A was obtained from Sandoz Ltd (Basel, Switzerland); picryl chloride was obtained from Nacalai Tesque Inc (Kyoto, Japan); 2,4,6-trinitrobenzenesulfonic acid hydrate (TNBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and mitomycin C were obtained from Sigma (St Louis, Mo); TRIZOL reagent was obtained from Sangon (Shanghai, China); M-MLV Reverse Transcriptase and oligo(dT)$_{18}$ primer were obtained from Promega (Madison, Wis); ELISA kit for murine IL-10 was obtained from Bender MedSystems (Vienna, Austria); glycylglycin was obtained from Nanjing Sunshine Biotechnology Co, Ltd (Nanjing, China), and SYBR Green I was obtained from Molecular Probes (Eugene, Ore). Sheep red blood cells (SRBCs) were obtained from Jiangning Center for Disease Control (Nanjing, China), and purified rat anti-mouse IL-10 mAb (JES5-2A5) and rat IgG1κ isotype control (R3-34) were obtained from BD PharMingen, San Diego, Calif).

Picryl chloride–induced contact hypersensitivity

Mice were sensitized by painting 0.1 mL of 1% picryl chloride in ethanol onto the shaved skin of their abdomens. Five days after sensitization, they were challenged on the right ear with 30 μL of 1% picryl chloride in olive oil. Ear swelling was evaluated by the difference in thickness between the right and left ears, as measured with an engineer's micrometer (0.001 mm; Mitutoyo Co, Tokyo, Japan) 24 hours after challenge. The negative control animals were normally sensitized and painted with olive oil alone when challenged. The positive control animals with contact hypersensitivity were given saline instead of drugs.

Histologic analysis

Formalin-fixed, paraffin-embedded ear tissue was sectioned at 5 μm in thickness, and the sections were stained with hematoxylin and eosin. Histopathologic scoring was done by using a range from 0 (no change) to 4 (most severe) to evaluate congestion, edema, and inflammatory cell infiltration.

Adoptive transfer of contact hypersensitivity

Donor BALB/c mice were immunized with picryl chloride, as described above. Six hours after challenge, single-cell suspensions were made from the draining regional lymph nodes (inguinal, brachial, and axillary), and the cell number was adjusted to 2.5 × 10^8 cells/mL in PBS. Then 200 μL of the suspensions were injected intravenously into each recipient mouse, followed by challenge in the ear with 30 μL of 1% picryl chloride. After 15, 19, and 24 hours, ear swelling in the recipient mice was evaluated.

Hapten-specific T-cell proliferation

Splenocytes isolated from BALB/c mice 5 days after sensitization were treated with 1.0 mM TNBS (water-soluble analog of picryl chloride) and 25 μg/mL mitomycin C for 30 minutes at 37°C. After washing 3 times in HBSS supplemented with 0.6% glycylglycin, the cells were used as stimulator cells (trinitrophenylated splenocytes). At the same time, lymph node cells from the above-described sensitized mice, from which the adherent cells had been removed, were used as responder cells. The 4 × 10^5 stimulator cells and 2 × 10^5 responder cells were cocultured for 72 hours. Then cell proliferation was examined by means of MTT assay. The stimulation index (SI) was calculated as follows:

$$Stimulation\ index = OD_{stimulated\ cells} / OD_{nonstimulated\ cells}.$$

Quantification of mRNA expression by means of RT-PCR and real-time PCR

Lymph node cells were isolated from mice. In some cases these cells were purified to T lymphocytes by mouse T-cell enrichment columns (R&D systems, Minneapolis, Minn). Total RNA was extracted with TRIZOL reagent. The cDNA synthesis reaction was performed from 2 μg of total RNA by using oligo(dT)$_{18}$ and M-MLV reverse transcriptase for first-strand cDNA synthesis. PCR primer sequences (Sangon) were as follows: β-actin, forward 5′-ACATCTGCTGGAAGGTGGAC and reverse 5′- GGTACCACCATGTACCCAGG; IL-10, forward 5′-GGTTGCCAAGCCTTATCGGA and reverse 5′-ACCTGCTCCACTGCCTTGCT; IFN-γ, forward 5′-CTTCTTCAGCAACAGCAAGGCGAAAA and reverse 5′- CCCCCA-