Effects of Anti-inflammatory Biflavonoid, Ginkgetin, on Chronic Skin Inflammation

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Ginkgetin, a biflavonoid from Ginkgo biloba leaves (Ginkgoaceae), was previously demonstrated to inhibit phospholipase A₂ and to suppress proinflammatory gene expression such as cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase. In this study, the effects of ginkgetin were examined on an animal model of chronic skin inflammation and proinflammatory gene expression. When topically applied to ICR mouse ear, ginkgetin (20—80 μg/ear/treatment) inhibited ear edema (22.8—30.5%) and prostaglandin E₂ production (30.2—31.1%) induced by multiple treatment of 12-O-tetradecanoylphorbol-13-acetate (TPA) for 7 consecutive days. By histological comparison, ginkgetin was also found to reduce epidermal hyperplasia. The expression of proinflammatory gene, interleukin-1β, was suppressed by ginkgetin. From the results, it is suggested that ginkgetin may be beneficial against chronic skin inflammatory disorders like atopic dermatitis.

Key words ginkgetin; biflavonoid; skin inflammation; gene expression

Although the etiology of chronic skin inflammatory disorders including psoriasis and atopic dermatitis (AD) is not clearly understood, many studies have shown that various proinflammatory enzymes/cytokines play an important role in these inflammatory diseases. For example, phospholipase A₂ (PLA₂) was highly expressed in some psoriatic tissues.¹ In addition to lipoxygenase products,² cyclooxygenase (COX) and prostaglandins (PG) were involved in dermal inflammation and skin wound healing.³ ⁴ Moreover, it was found that high concentrations of cytokines, such as tumor necrosis factor (TNF)-α, and adhesion molecules, including intercellular adhesion molecule (ICAM)-1, were deeply involved in the regional site of chronic skin inflammation.⁵ ⁶ Recent studies have also indicated that inducible nitric oxide synthase (iNOS) may be associated with psoriasis, and its reaction product, NO, is involved in various skin disorders.⁷ ⁸ Therefore, it is reasonably thought that an interference of activity and/or expression of these proinflammatory molecules may result in attenuation of chronic skin inflammatory syndromes, and the agents acting on these points may give beneficial effects.

Biflavonoids are flavonoid–flavonoid dimers and some of them possess anti-inflammatory activity. For instance, several biflavonoids including amentoflavone, ginkgetin and isoginkgetin inhibited histamine release.⁹ Amentoflavone and tetrahydroamentoflavone were found to inhibit COX-1 and/or COX-2.¹⁰ ¹¹ Some biflavonoids were revealed to suppress the expression of proinflammatory molecules such as COX-2 and iNOS.¹² ¹³ These previous investigations have shown the potential of certain biflavonoids for anti-inflammatory agents. In particular, ginkgetin (Fig. 1) mainly found in Ginkgo biloba leaves (Ginkgoaceae) was previously demonstrated as an inhibitor of group II A sPLA₂ inhibitor¹⁴ and cPLA₂.¹⁵ In addition, ginkgetin was reported to suppress COX-2 expression from lipopolysaccharide (LPS)-treated macrophages without affecting COX-2 activity, and in vivo study has also revealed that ginkgetin inhibited COX-2 expression and PGE₂ production from mouse skin induced by 3-d treatment of 12-O-tetradecanoylphorbol-13-acetate (TPA).¹⁶ All these findings strongly suggest that ginkgetin may show an inhibition against inflammatory disorders, especially skin inflammation by topical application. Thus, for further characterization of the pharmacological property, the effects of ginkgetin were examined on an animal model of chronic skin inflammation and proinflammatory gene expression in the present investigation.

MATERIALS AND METHODS

Chemicals  TPA and prednisolone were obtained from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.). Ginkgetin was isolated from the methanol extract of Ginkgo biloba leaves according to the previously described procedure.¹⁷ The purity was >95% based on HPLC analysis.

Animals  Male ICR mice (4 weeks, specific pathogen-free) were obtained from Orient Co. (Korea). Animals were fed with laboratory chow (Purina Korea) and water ad libitum. They were acclimatized in an animal facility (KNU) under the conditions of 20—22 °C, 40—60% relative humidity and 12 h/12 h (light/dark) cycle at least 7 d prior to experiment.

Chronic Skin Inflammation  On day of the experiment (day 1), TPA (1 μg/20 μl acetone) was applied to the inner and outer surfaces of mouse ear for inducing a chronic type of skin inflammation according to the original procedure¹⁸ with some modification. Test compounds dissolved in oil-based vehicle were topically applied to the same site (20 μl) at 1 and 12 h after TPA treatment. TPA-treated control group only received TPA and vehicle. On day 2—6, the same treat-

Fig. 1. Chemical Structure of Ginkgetin

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ment regimen was carried out with TPA and test compounds. On day 7, TPA was applied, and one hour later, test compounds were treated. Three randomly selected mice/group were sacrificed by cervical dislocation 3 h after final treatment of test compounds. Ears were removed and stored in RNA stabilization reagent (Qiagen, Germany) at −20°C for reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. For determination of anti-inflammatory activity, ear thickness of the remaining five mice/group was measured using an engineering gauge (Mitutoyo, Japan) 5 h after final treatment of test compounds. Immediately after, in order to determine PGE2 concentration and to prepare histological samples, ears were removed. For evaluation of the effects on normal skin, test compounds in the vehicle were treated topically to ears of mice using the same treatment schedule as above without TPA treatment. In this case, the control group received acetone and vehicle only. After 7 d, ear thickness of the test compound-treated groups was measured, but no noticeable difference was observed between the treated and the control groups.

**RT-PCR Analysis** After cutting into small pieces, ear samples were homogenized in RLT buffer containing 1% β-mercaptoethanol for 30 s using Polytron homogenizer. Total RNA was extracted using RNeasy mini kit (Qiagen) according to the supplier’s protocol. The concentration of RNA content was determined by measuring the absorbance at 260 and 280 nm. cDNAs were synthesized using RT reaction at 42°C, 50 min and 99°C, 5 min in Gene Cycler thermal cycler (Bio-Rad). Primers were synthesized on the basis of the repeated mouse cDNA sequence for COX-1, COX-2, interleukin-1β (IL-1β), TNF-α, iNOS, ICAM-1, fibronectin and G3PDH. The primer sequences used for PCR were as follows: COX-1 sense, 5'-TGC ATG TGG TGT TGG TCA TCA A-3', antisense, 5'-CAG TAA GAC AGA CCC GTC ATC TCC A-3', 450 bp; COX-2 sense, 5'-ACT CAT TCA GTT TGT TGA GTC ATT C-3', antisense, 5'-TTT GAT TAG TAC TGT AGG GTT AAT G-3'; IL-1β sense, 5'-TGC AGT AGT CCA CAT CGC AGC TCA C-3', antisense, 5'-GTG CTG CCT AAT GAT CCC TTG AAT C-3', 387 bp; TNF-α sense, 5'-ACA AGC GTG TAG TAC GGT ACG ACG-3', antisense, 5'-TCC AAA GTA GAC GTG CCC-3', 428 bp; iNOS sense, 5'-CCC TTC CGA AGT TTT TGG CAG CAG C-3', antisense, 5'-GGT GTG TCA AGG TCC GTG GCC ACC-3', 469 bp; ICAM-1 sense, 5'-TGG GAG GAT CAT AAA CGA AGC-3', antisense, 5'-AAC ATA AGA GGC TGC CAT AAC-3', 471 bp; fibronectin sense, 5'-GCA ACG TGT TAT GAC GAG-3', antisense, 5'-CTA ACG GCA TGA AGC ACT CA-3', 253 bp; G3PDH sense, 5'-TGA AGG TCG TGT TGA ACG CAT TTG GC-3', antisense, 5'-CAT GGT GGC CAT GAG TGC CAC CAC-3', 983 bp. PCR was carried out for 25—30 cycles under saturation, in 25 μl reaction mixture. After amplification, 5 μl of reaction mixture was analyzed on 1.5% agarose gel electrophoresis. The bands were visualized by ethidium bromide staining for 10 min. The band density was quantified by densitometric scanning using SigmaGel (Version 1.0, Jandel Sci.). The signal intensities were normalized by comparison with that of G3PDH and represented as relative ratios.

**Measurement of PGE2 Concentration** As an index of skin PLA2 and/or COX activity, PGE2 concentration was measured essentially following the previously described procedure.\(^1\)

In brief, the biopsies were obtained by 4 mm punch from four randomly selected ear samples/group. Then, they were homogenized in 100 μl phosphate buffer (pH 7.4) containing 1 mM EDTA and 10 μM indomethacin. After centrifugation at 1500 g for 10 min, 50 μl of citrate buffer (pH 3.5) was added to the supernatant. The mixture was centrifuged again at 2500 g for 10 min. The resulting supernatant was applied to a 6 ml Sep-Pak C18 cartridge (Waters Associate, U.S.A.) and eluted with 5 ml ethyl acetate containing 1% methanol. The eluent was dried under N2 stream and PGE2 concentration was measured with an ELISA kit (Cayman Chem.) according to the manufacturer’s instruction.

**Histology** Ear samples were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned and stained with haematoxylin and eosin (H&E) based on the standard procedures.

**Statistical Analysis** All results were represented as arithmetic mean±S.D. One-way ANOVA and Student’s t-tests were used for evaluation of a statistical significance.

**RESULTS**

Seven day multiple treatment of TPA on ICR mouse ear induced a chronic type of skin inflammation, characterized by edema, epidermal hyperplasia and infiltration of inflammatory cells (Figs. 2a, b). By histological comparison, ginkgetin was found to considerably reduce these responses (Fig. 2c). Prednisolone used as a reference drug reduced these responses more profoundly (Fig. 2d). When the ear thickness was measured, more than twice increase was observed compared with those of non-treated mice (0.475±0.031 mm from 0.206±0.005 mm) (Fig. 3). By topical application, ginkgetin significantly inhibited ear edema (22.8, 30.5% inhibition at 20, 80 μg/ear/treatment, respectively) (Fig. 3a). Prednisolone (10 μg/ear/treatment) showed potent inhibition of ear edema (66.3%). Using the ear biopsies, PG levels were checked. As expected, TPA treatment drastically increased PGE2 concentration in the lesion (24.8±2.1 ng/biopsy) from the basal level (3.4±0.3 ng/biopsy). Ginkgetin moderately reduced PGE2 concentration (30.2—31.1%), while prednisolone showed a slightly higher inhibition of PGE2 production (36.5%).

For elucidating the effects on proinflammatory gene expression, RT-PCR was employed using the ear biopsies. TPA treatment considerably induced the expression of COX-2 and IL-1β genes (Fig. 4). Other inducible genes of ICAM-1 and TNF-α were very weakly induced whereas iNOS mRNA was not detected. The constitutive genes, COX-1 and fibronectin, were constantly expressed as expected, but there was some increase of mRNA expression by 7-d TPA treatment. Under this condition, ginkgetin dose-dependently inhibited IL-1β expression among the inducible genes tested, but not statistically significant (16.6, 50.9% inhibition at low and high dose treatment, respectively). COX-2 expression was weakly reduced only by high dose treatment of ginkgetin (13.7% inhibition). The changes of ICAM-1 and TNF-α gene expression by ginkgetin were not meaningful since expression levels of these two genes were too low. On the other hand, prednisolone potently inhibited COX-2 and IL-1β expression (75.7, 95.7% inhibition, respectively).

For toxicity evaluation, ginkgetin was applied to the dorsal
area of SKH-1 hairless mice (400 μg/mouse/d, 5 d a week). After 3 months, any apparent toxicity including appearance difference and differences of body weights and major organ weights was not found (data not shown). All these findings indicate that ginkgetin possesses inhibitory activity against chronic skin inflammation and may be safely used in long-term topical treatment.

DISCUSSION

This investigation has clearly proven that ginkgetin inhibited the inflammatory responses of an animal model of chronic skin inflammation. Depending on animal models of skin inflammation employed, there are substantial differences in the nature of inflammation produced. For instance, phenol-treated contact dermatitis in mice produced an acute inflammation accompanied by dermal edema, and an animal model of delayed hypersensitivity induced an infiltration of inflammatory cells in the lesion.19,20) Multiple TPA treatment used in this study, on the other hand, provoked the characteristic changes such as edema and epidermal keratinocyte proliferation. These results were well matched with other’s finding.21) In particular, ginkgetin reduced the epidermal hyperplasia.

Previously, it was found that multiple treatment of TPA for 3 consecutive days (3-d model) potently induced several proinflammatory genes including COX-2, IL-1β, TNF-α and ICAM-1 on ICR mouse ear.22) Compared to 3-d model, COX-2 mRNA expression was weaker, and the degree of COX-2 and IL-1β expression had larger variation within the animals of the same group. The expression levels of ICAM-1 and TNF-α expression were minimum in 7-d model, while IL-1β gene expression was prominent on both models. It is thought that a shift to chronic response in 7-d model from 3-d model may provide these changes, suggesting that IL-1β expression is likely more important than those of COX-2, ICAM-1 and TNF-α. Ginkgetin was found to inhibit IL-1β expression. However, it is not certain why there was some increase of COX-1 and fibronectin mRNA levels by TPA treat-
and COX-2 activities.\textsuperscript{16} Therefore, an inhibition of COX-2 by ginkgetin was previously found to show no effect on COX-1 concentration,\textsuperscript{17} indicating that the meaningful reduction of COX-2 expression. Furthermore, contrary, in the present 7-d model, ginkgetin did not show any reduction of PGE\textsubscript{2} concentration (Fig. 4a) or induction and PGE\textsubscript{2} production in the skin lesion.\textsuperscript{22} On the other hand, the inhibition of PLA\textsubscript{2} and/or inhibition of IL-1\textbeta expression by ginkgetin, this reduction of PGE\textsubscript{2} concentration by ginkgetin treatment. In- stead, it is speculated that the some reduction of PGE\textsubscript{2} concentration may be at least partly due to PLA\textsubscript{2} inhibition as well as inhibiting PLA\textsubscript{2}. Ginkgetin may be beneficial against chronic skin inflammatory disorders such as AD by topical application.

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