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Inhibition of Chronic Skin Inflammation by Topical Anti-inflammatory Flavonoid Preparation, Ato Formula®

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Flavonoids are known as natural anti-inflammatory agents. In this investigation, an anti-inflammatory potential of new topical preparation (SK Ato Formula®) containing flavonoid mixtures from *Scutellaria baicalensis* Georgi roots and *Ginkgo biloba* L. leaves with an extract of *Gentiana scabra* Bunge roots was evaluated in an animal model of chronic skin inflammation. Multiple 12-O-tetradecanoylphorbol-13-acetate treatments for 7 consecutive days on ICR mouse ear provoked a chronic type of skin inflammation: dermal edema, epidermal hyperplasia and infiltration of inflammatory cells. When topically applied in this model, this new formulation (5-20 μL/ear/treatment) reduced these responses. Furthermore, it inhibited prostaglandin E_2 generation (17.1-33.3%) and suppressed the expression of proinflammatory genes, cyclooxygenase-2 and interleulin-1β in the skin lesion. Although the potency of inhibition was lower than that of prednisolone, all these results suggest that Ato Formula® may be beneficial for treating chronic skin inflammatory disorders such as atopic dermatitis.

Key words: Flavonoid, Skin inflammation, Ato Formula®, Prostaglandin, Interleukin

INTRODUCTION

To present, it is still not feasible to successfully treat chronic skin inflammatory disorders including psoriasis and atopic dermatitis (AD) despite use of a variety of drugs such as steroidal anti-inflammatory drugs (SAID). Therefore, there is a need for new agents having different cellular action mechanism(s) from those of conventional drugs. Many studies have shown that various proinflammatory enzymes/cytokines play an important role in these inflammatory diseases. They include phospholipase A_2 (PLA_2), cyclooxygenases (COX), lipoxygenases (LOX) and cytokines such as tumor necrosis factor (TNF)-α (Andersen *et al.*, 1994; Fogh and Kragballe, 2000; LaDucca and Caspari, 2001). Thus, it is reasonable to think that an interference of activity and/or expression of these proinflammatory molecules may inhibit chronic skin inflammation, and the agents acting on these points would give beneficial effects.

Many flavonoids from plant origin are anti-inflammatory agents and their activities including inhibition of arachidonate metabolizing enzymes and suppression of expression of proinflammatory molecules have been proved *in vitro* and *in vivo* (Middleton *et al.*, 2000; Kim *et al.*, 2004). Among these derivatives, certain compounds such as flavones from *Scutellaria baicalensis* Georgi roots possess high anti-inflammatory activity and favorable cellular action mechanism(s) against chronic skin inflammatory disorders when topically applied. SK Ato Formula® is a topical anti-inflammatory agent targeted for a treatment of chronic skin inflammation, especially AD. This new preparation is mainly a mixture of flavonoid fractions from *S. baicalensis* roots and *Ginkgo biloba* L. leaves with an extract of *Gentiana scabra* Bunge roots. The present study was carried out to establish anti-inflammatory potential of this topical formulation against an animal model of chronic skin inflammation.

MATERIALS AND METHODS

Chemicals

12-O-Tetradecanoylphorbol-13-acetate (TPA) and prednisolone were obtained from Sigma-Aldrich Co. Ato Formula® is basically a mixture of cream base and flavonoid

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fractions from *S. baicalensis* roots (0.15%, w/w) and *G. biloba* leaves (0.10%, w/w) with an extract of *G. scabra* roots (0.20%, w/w). Ato Formula® and cream base were supplied by SK Chemicals (Seoul, Korea). As a reference drug, prednisolone cream prepared by the same company was used throughout this study.

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 a specific pathogen-free 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 days prior to experiment.

Multiple TPA-induced chronic skin inflammation in mice

For an animal model of chronic skin inflammation, multiple treatment of TPA for 7 days using eight mice/group was carried out according to the original procedure (Stanley *et al.*, 1991) with some modification. In brief, TPA (1 μg/20 μL acetone) was applied to the inner and outer surfaces of mouse ear on day 1. Test compounds were topically applied to the same site (5-20 μL/ear) at 1 and 12 h after TPA treatment. On day 2-6, the same treatment regimen was carried out with TPA and test compounds. On day 7, TPA was applied, and one hour later, test compounds were treated. Three mice per 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 was measured using an engineering gauge (Mitutoyo, Japan) 5 h after final treatment of test compounds. Immediately after, in order to check prostaglandin E_2 (PGE_2) concentration and to prepare histological samples, ears were removed.

RT-PCR analysis

All procedures of RNA extraction from ear biopsies and RT-PCR were essentially same as previously described (Chi *et al.*, 2003). The primer sequences used for PCR were as follows: COX-1 sense, 5'-TGC ATG TGG CTG TGG ATG TCA TCA A-3', antisense, 5'-CAC TAA GAC AGA CCC GTC ATC TCC A-3', 450 bp; COX-2 sense, 5'-ACT CAC TCA GTT TGT TGA GTC ATT C-3', antisense, 5'-TTT GAT TAG TAC TGT AGG GTT AAT G-3', 583 bp; interleukin (IL)-1β sense, 5'-TGC AGA GTT CCC CAA CTG GTA CAT C-3', antisense, 5'-GTG CTG CCT AAT GTC CCC TTG AAT C-3', 387 bp; TNF-α sense, 5'-ACA AGC CTG TAG CCC ACG-3', antisense, 5'-TCC AAA GTA GAC CTG CCC-3', 428 bp; inducible nitric oxide synthase (iNOS) sense, 5'-CCC TTC CGA AGT TTC TGG CAG CAG C-3', antisense, 5'-GGC TGT CAG AGC CTC GTG GCT TTG G-3', 469 bp; intercellular adhesion molecule (ICAM)-1 sense, 5'-TCG GAG GAT CAC AAA CGA AGC-3', antisense, 5'-AAC ATA AGA GGC TGC CAT CAC G-3', 471 bp; fibronectin sense, 5'-GCA ACG TGT TAT GAC GAT GG-3', antisense, 5'-CTA ACG GCA TGA AGC ACT CA-3', 253 bp; glyceladehyde-3-phosphate dehydrogenase (G3PDH) sense, 5'-TGA AGG TCG GTG TGA ACG GAT TTG GC-3', antisense, 5'-CAT GTA GGC CAT GAG GTC CAC CAC-3', 983 bp. After amplification and 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 prostaglandin E_2 (PGE_2) concentration

As an index of skin PLA_2 and/or COX activity, PGE_2 concentration in ear biopsies was measured essentially following the previously described procedure (Chi *et al.*, 2003). Briefly, from the biopsies, homogenate was obtained and centrifuged. The resulting supernatant was applied to a 6 mL Sep-Pak C_{18} cartridge (Waters Associate, U.S.A.) and eluted with 5 mL ethyl acetate containing 1% methanol. The eluent was dried under N_2 stream and PGE_2 concentration was measured with an ELISA kit (Cayman Chem.) according to the manufacturer's instruction.

Histology

The 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. Student *t*-test was used for evaluation of statistical significance.

RESULTS AND DISCUSSION

Multiple TPA treatment on mouse ear for 7 consecutive days provoked a chronic type of skin inflammation. Prominent epidermal hyperplasia occurred along with dermal edema and infiltration of inflammation-related cells (Fig. 1b). By histological comparison, Ato Formula® was found to reduce these inflammatory changes. Especially, a high dose treatment of this new preparation (20 μL/ear/treatment) considerably inhibited epidermal hyperplasia,

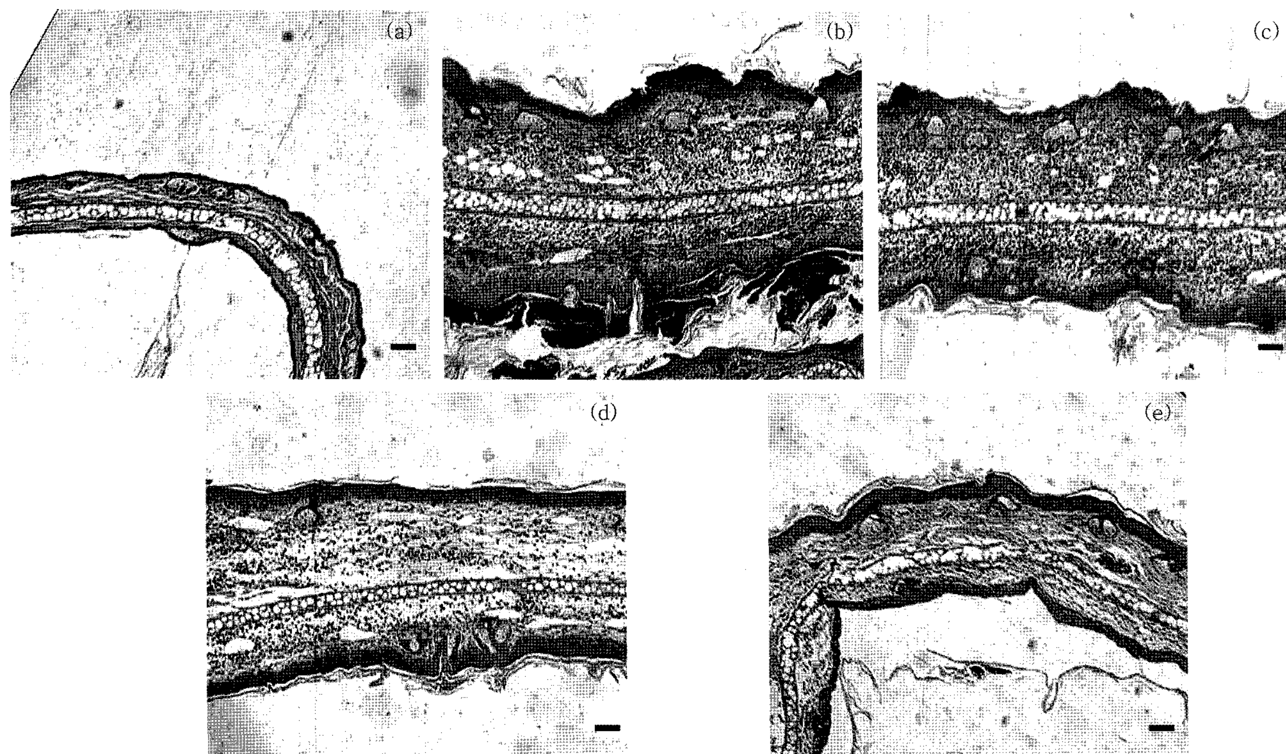

Fig. 1. Cross sections of ICR mouse ear. (a) Non-treated, (b) TPA, (c) TPA + Ato Formula® (5 μL/ear/treatment), (d) TPA + Ato Formula® (20 μL/ear/treatment), (e) TPA + prednisolone (10 μg/ear/treatment). The scale bar represented 100 μm. Note: Numerous inflammatory cells including neutrophils and monocytes were infiltrated in the dermal area of TPA-treated ear (b). The apparent reduction of the recruitment of these cells was observed in (d) and (e). The keratinocyte epidermal layers increased to 3-6 in (b) from the basal level of 1-2. Treatment of Ato Formula® and prednisolone reduced epidermal hyperplasia as in (d) and (e).

dermal edema and inflammatory cell infiltration (Fig. 1d). A further reduction of these parameters was observed in the prednisolone-treated skin (Fig. 1e). Ato Formula® (5-20 μL/ear/treatment) also inhibited ear edema and PGE_2 production induced by TPA treatment (Table I). Prednisolone (10 μg/ear/treatment) showed a potent inhibition of ear edema and PGE_2 production as expected. Using RT-PCR analysis, the expression levels of several proinflammatory genes in ear biopsies were examined. TPA treatment

Table I. Inhibition of 7 day TPA-induced ear edema by Ato Formula®

Grous	Dose/ear/ treatment	Ear thickness[a] increased (mm)	PGE_2[b] (ng/biopsy)
TPA	-	0.306 ± 0.038	58.7 ± 16.5
Ato Formula®	5 μL	0.224 ± 0.109 (26.8)	49.2 ± 7.1 (17.1)
	20 μL	0.200 ± 0.048* (34.6)	40.2 ± 4.7 (33.3)
Prednisolone	10 μg	0.112 ± 0.028* (63.4)	24.0 ± 1.4* (62.6)

[a]n = 5, [b]n = 2, The values in parenthesis represent % inhibition, which was calculated as [(TPA-control) – (test-control)/(TPA-control)] × 100. The control group showed ear thickness increase and PGE_2 concentration of 0.006 ± 0.013 mm and 3.3 ± 0.0 ng/biopsy, respectively. The ear thickness of the control group on day 1 was 0.201 ± 0.016 mm. *: P < 0.01, Significantly different from the TPA-treated group.

induced COX-2 and IL-1β genes (Fig. 2). ICAM-1 and TNF-α genes were very weakly induced, while iNOS gene expression was not detected (data not shown). On the other hand, COX-1 and fibronectin were constitutively expressed. A topical application of Ato Formula® substantially inhibited IL-1β expression whereas COX-2 expression was significantly reduced only by a low dose treatment (5 μL/ear/treatment). In comparison, prednisolone potently inhibited COX-2 and IL-1β expression. It is meaningful to note that TPA treatment somewhat increased the expression of the constitutive genes, COX-1 and fibronectin, and a high dose treatment of Ato Formula® further enhanced the expression of these genes. Although the reason for the increased expression of COX-1 and fibronectin by TPA treatment is not clear, a further increase of COX-1 and fibronectin expression by a high dose treatment of Ato Formula® may reflect the enhancement of cellular activity in the skin lesion. And this speculation may be related with the finding of less reduction of COX-2 and IL-1β expression by a high dose treatment of Ato Formula®.

In order to check long-term toxic effect on normal skin, Ato Formula® was applied to the dorsal area of SKH-1 hairless mice (100 mg/mouse/day, 5 days a week). After 3 months of trial, skin histological samples were prepared

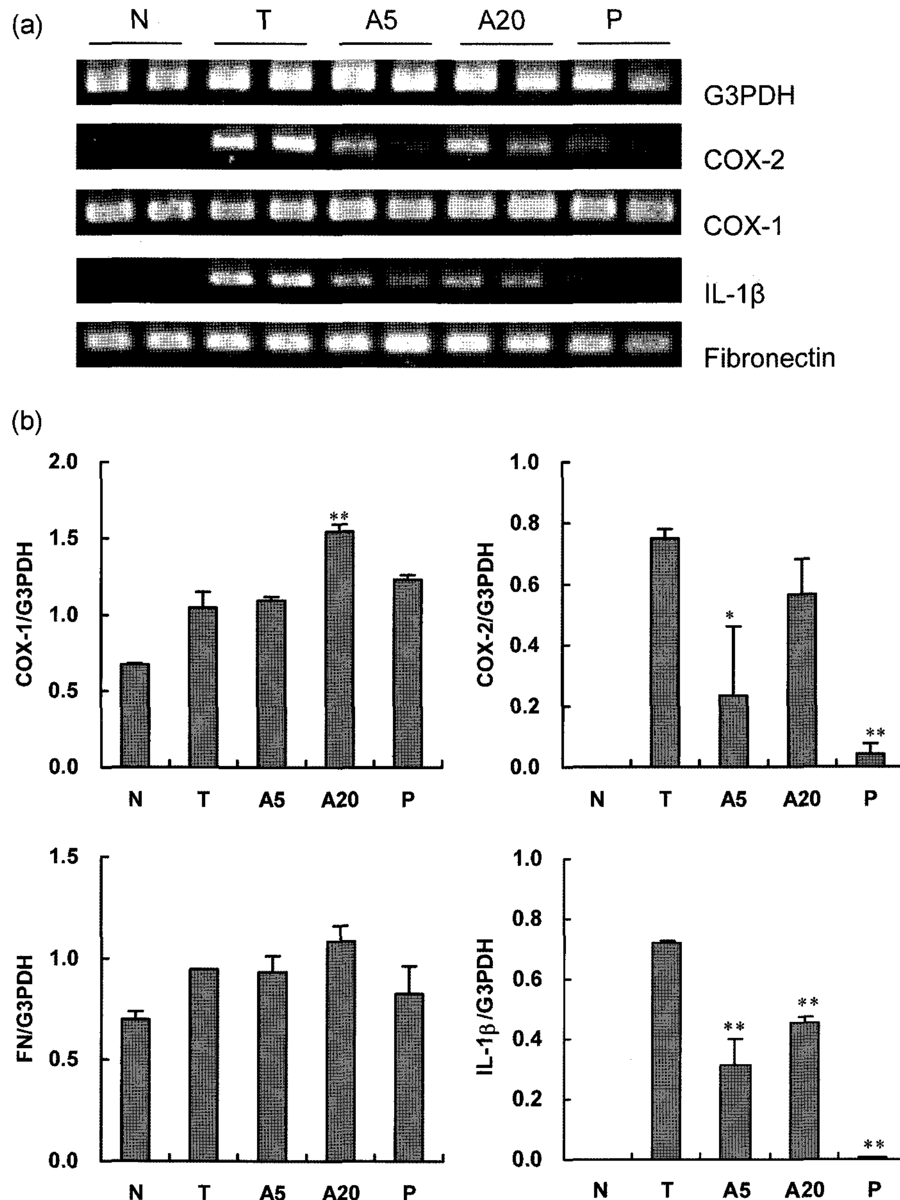

Fig. 2. Effects on proinflammatory gene expression. (a) RT-PCR analysis. The results from three randomly selected ear samples were shown here. N: Non-treated, T: TPA, A: TPA + Ato Formula® (5 and 20 μL/ear/treatment), P: TPA + prednisolone (10 μg/ear/treatment). (b) Density ratios. *: $P < 0.1$, **: $P < 0.05$, Significantly different from the TPA-treated group (n = 3).

and no abnormal change was observed. Any apparent toxic effect on the changes of body weights and major organ weights was not found (data not shown).

By topical treatment of Ato Formula®, two biochemical parameters were substantially affected: arachidonate metabolism and proinflammatory gene expression. Although the detailed mechanisms of PGE_2 generation in this animal model need to be further studied, it is generally thought that mainly a cytosolic isoform of PLA_2 ($cPLA_2$) generates arachidonic acid (AA), which is further metabolized to PGs by COX-2, induced by TPA treatment. Previously, some flavonoids such as wogonin and quercetin were found to inhibit COX-2 (Chi *et al.*, 2001a and b). And certain flavonoids, especially biflavonoids including ochnaflavone and ginkgetin, were revealed as PLA_2 inhibitors (Chang *et al.*, 1994; Gil *et al.*, 1997; Kim *et al.*, 2001). Since wogonin, quercetin and biflavonoids including ginkgetin are major ingredients of Ato Formula®, it is suggested that the reduction of PGE_2 production by this new formulation may be partly due to the effects on COX-2, and/or partly due to PLA_2 inhibition in the skin. In addition, Ato Formula® inhibited IL-1β expression considerably with less effect on COX-2 expression in ear skin. Several flavonoid derivatives including quercetin, wogonin and ginkgetin are the known

regulators of proinflammatory gene expression *in vitro* and *in vivo* (Kim *et al.*, 2004). Therefore, it is reasonably thought that these flavonoid components in Ato Formula® may contribute to the suppression of IL-1β and COX-2 expression in the skin lesion.

One of the ingredients in this new formulation is the root of *G. scabra*, which is distributed in China, Japan and Korea. It has been used as anti-inflammatory, analgesic and antifungal agent (Natural Product Research Institute, 1998). The alkaloids such as gentianine and gentianidine and the iridoids such as sweroside and gentiopicroside were reported as its constituents (Jensen and Schripsema, 2002). Recently, one of these compounds, gentianine, was found to suppress TNF-α and IL-6 production from LPS-treated rats (Kwak *et al.*, 2005). These previous findings strongly suggest that the *G. scabra* extract may also contribute, at least in part, to the inhibitory activity of Ato Formula® against skin inflammation.

In conclusion, Ato Formula® inhibits chronic skin inflammation, and its anti-inflammatory activity may be exerted at least in part by inhibition of eicosanoid generating enzymes such as PLA_2 and COX-2, and/or in part by down-regulation of proinflammatory gene expression. As anti-inflammatory agents having different cellular action mechanism(s) from those of conventional drugs are desirable, Ato Formula® may have a potential of new topical preparation targeted for chronic skin inflammatory disorders such as AD.

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