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

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(Received February 19, 2006)

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 E₂ generation (17.1-33.3%) and suppressed the expression of proinflammatory genes, cyclooxygenase-2 and interleukin-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 A₂ (PLA₂), cyclooxygenases (COX), lipooxygenases (LOX) and cytokines such as tumor necrosis factor (TNF)-α (Andersen et al., 1994; Fogh and Kragballe, 2000; LaDuca 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
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 E₂ (PGE₂) 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 CTT TGG ATG TCA TCA A-3', antisense, 5'-CAG 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 GAT AAT G-3'; 583 bp; interleukin (IL)-1β sense, 5'-TGC AGA GGT 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 CCA AGT TTT TGG CAG CAG C-3'; antisense, 5'-GGC TGT CAG AGC CTC GTG CTT 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 AGC TGT TAT GAC GAT GG-3'; antisense, 5'-CTA AGC GCA TGA AGC ACT CA-3'; 253 bp; glyceraldehyde-3-phosphate dehydrogenase (G3PDH) sense, 5'-TGA AGG TCG TGT 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.).

**Measurement of prostaglandin E₂ (PGE₂) concentration**

As an index of skin PLA₂ and/or COX activity, PGE₂ 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 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**

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,