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Inhibition of Contact Dermatitis in Animal Models and Suppression of Proinflammatory Gene Expression by Topically Applied Flavonoid, Wogonin

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Wogonin (5,7-dihydroxy-8-methoxyflavone) is a down-regulator of cyclooxygenase-2 and inducible nitric oxide synthase expression, contributing to anti-inflammatory activity *in vivo*. For further characterization of modulatory activity on proinflammatory gene expression *in vivo*, the effect of wogonin was examined in this experiment using animal models of skin inflammation. By topical application, wogonin inhibited an edematic response as well as proinflammatory gene expression against contact dermatitis in mice. Wogonin inhibited ear edema (19.4-22.6%) at doses of 50-200 μg/ear and down-regulated interleukin-1β induction (23.1%) at 200 μg/ear in phenol-induced simple irritation. Wogonin (2×50-2×200 μg/ear) also inhibited edematic response (51.2-43.9%) and down-regulated proinflammatory gene expression of cyclooxygenase-2, interleukin-1β, interferon-γ, intercellular adhesion molecule-1 and inducible nitric oxide synthase with some different sensitivity against picryl chloride-induced delayed hypersensitivity reaction. All these results clearly demonstrate that wogonin is a down-regulator of proinflammatory gene expression in animal models of skin inflammation. Therefore, wogonin may have potential for a new anti-inflammatory agent against skin inflammation.

Key words: Flavonoid, Wogonin, Inflammation, Gene expression, Cyclooxygenase, Nitric oxide synthase, Interleukin, Interferon

INTRODUCTION

Various flavonoids from plants show anti-inflammatory activity *in vitro* and *in vivo*. One of their cellular action mechanisms is an inhibition of inflammation-related enzymes (Middleton *et al.*, 2000). Examples of target enzymes are phospholipase A_2 (PLA_2), cyclooxygenases (COX) and lipoxygenases (LOX), producing arachidonic acid and proinflammatory eicosanoids. It has been also revealed that some flavonoids, especially flavone derivatives, can modulate the expression levels of proinflammatory enzymes and cytokines including COX-2 and inducible nitric oxide synthase (iNOS) *in vitro* (Krol *et al.*, 1995; Soliman and Mazzio, 1998; Liang *et al.*, 1999; Kim *et al.*, 1999; Raso *et al.*, 2001). These modulating activities of proinflammatory gene expression may contribute to the anti-inflammatory action exerted by certain flavonoids. Indeed, when peritoneally administered, several flavonoid derivatives such as luteolin and quercetin were shown to inhibit proinflammatory gene expression in experimental animal models (Takahashi *et al.*, 2001; Kotanidou *et al.*, 2002).

Among the flavonoid derivatives examined so far, wogonin (Fig. 1) has been found to be the most potent in suppressing COX-2 and iNOS expression from bacterial lipopolysaccharide (LPS)/cytokine-treated mouse macrophages or macrophage-like cell line, RAW 264.7 cells (Wakabayashi, 1999; Kim *et al.*, 1999; Chi *et al.*, 2001). This compound was also proved to suppress COX-2 induction and reduce prostaglandin E_2 production on 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-treated mouse skin by topical application (Park *et al.*, 2001). A further study revealed that wogonin potently inhibited COX-2 and tumor necrosis factor (TNF)-α expression with less effect on intercellular adhesion molecule (ICAM)-1 and interleukin (IL)-1β expression in a sub-chronic skin inflammation model provoked by multiple TPA treatment (Chi *et al.*,

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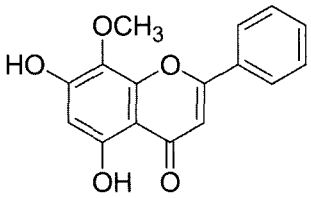

Fig. 1. Chemical structure of wogonin

2003). These previous investigations strongly suggest that wogonin exerts anti-inflammatory activity against skin inflammation, at least in part, by suppressing pro-inflammatory gene expression. For further characterization of the pharmacological properties of wogonin, it is necessary to elucidate its effect on other animal models of skin inflammation. Therefore, in this study, the effect of wogonin as a representative flavonoid on several pro-inflammatory gene expression was examined in mouse models of contact dermatitis, and wogonin's potential use as a new agent against skin inflammation was discussed.

MATERIALS AND METHODS

Chemicals

TPA and prednisolone were obtained from Sigma-Aldrich Co. Picryl chloride was a product of Nacalai Tesque Inc. (Japan). Wogonin was isolated from the methanol extract of Scutellaria radix according to the previously described procedure (You *et al.*, 1999). The purity of wogonin was determined by HPLC analysis and proved to be >95%.

Animals

Male specific pathogen-free ICR mice (18-22 g) were obtained from Daihan Biolink Co. (Emsung, Korea). Animals were maintained and acclimatized with Purina laboratory chow and water *ad libitum*, at least 7 days prior to experiments under the conditions of 21±1°C, 40-60% relative humidity and 12 h/12 h (light/dark) cycle.

Phenol-induced contact dermatitis

Simple skin irritation was induced by phenol treatment. Briefly, 10% phenol in acetone (20 μL) was topically smeared on the right ear of a mouse (five mice/group). Test compounds in an oil-based vehicle (20 μL/ear) were topically applied 5 min after phenol treatment. Control group received only the same amount of vehicle. Two hours later after phenol treatment, ear thickness was measured using an engineering gauge (Mitutoyo Co., Japan), and immediately after, animals were sacrificed. Ears were excised and stored at -70°C for reverse transcription-polymerase chain reaction (RT-PCR) analysis.

Picryl chloride-induced delayed hypersensitivity

Hair of abdomen of mice was cut and 7% picryl chloride in acetone (100 μL/mouse) was smeared to sensitize the animals for the elicitation phase group. For the control group animals and the induction phase group animals, acetone (100 μL) was applied instead of picryl chloride. Seven days later, the elicitation phase of delayed hypersensitivity (P-P) was induced by application of 1% picryl chloride in acetone (20 μL/ear) to the right ears of the sensitized mice. To obtain the induction phase reaction (A-P), 1% picryl chloride (20 μL/ear) was applied to the right ears of acetone-treated mice. For the control group (A-A, control), only acetone (20 μL) was applied to the right ears of acetone-treated mice. After 24 h, ear thickness was measured, and mice were sacrificed for RT-PCR analysis. Test compounds in vehicle (20 μL/ear) were applied to the right ears of mice 1 h after initial treatment with sensitizer or acetone. The same amounts of test compounds in vehicle (20 μL/ear) were treated again on the same site 1 h after final picryl chloride or acetone treatment.

RT-PCR analysis

All procedures including reverse transcription were carried out according to the previous report (Chi *et al.*, 2003). In brief, after cutting into small pieces, three randomly selected ear samples from each group were homogenized for 30 sec in RLT buffer containing 1% β-mercaptoethanol. Total RNA was extracted with RNeasy mini kit (Qiagen) and the concentration of RNA content was determined by measuring the absorbance at 260 and 280 nm. cDNAs were synthesized using a RT reaction at 42°C for 50 min and then at 99°C for 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, IL-1β, TNF-α, iNOS, ICAM-1, interferon (IFN)-γ, fibronectin and G3PDH. 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; 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; 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; 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; IFN-γ sense, 5'-GCT GTT TCT GGC TGT TAC TG-3', antisense, 5'-GAC TCC TTT TCC

GCT TCC TG-3', 495 bp; fibronectin sense, 5'-GCA ACG TGT TAT GAC GAT GG-3', antisense, 5'-CTA ACG GCA TGA AGC ACT CA-3', 253 bp; 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. 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. And the band densities were semi-quantitatively measured by densitometric scanning using SigmaGel (Version 1.0, Jandel Sci.). The signal intensities were normalized by comparing with those of G3PDH and represented as relative ratios.

Statistical analysis

All results were represented as arithmetic mean±SD. One way ANOVA test was used for statistical significance.

RESULTS

In order to produce simple irritation, 10% phenol was applied to the ears of mice. Phenol treatment increased ear thickness to 0.27±0.01 mm from the control value, 0.19±0.01 mm (n = 5) in 2 h (Fig. 2). Under this condition, wogonin weakly inhibited the increase of ear thickness (edematic response), but not to a statistically significant extent (19.4% and 22.6% inhibition at 50 and 200 μg/ear, respectively). Prednisolone was used as a reference anti-inflammatory drug and showed 32.3% inhibition of ear edema at 50 μg/ear. When RT-PCR analysis was carried out with intact ear skin (control), mRNAs of the constitutive genes such as COX-1, fibronectin and G3PDH were detected, while the inducible proinflammatory genes including COX-2, IL-1β, TNF-α, IFN-γ, ICAM-1 and iNOS were not expressed (Fig. 3). On the other hand, phenol treatment considerably induced IL-1β mRNA gene only among the inducible genes checked. It is interesting to note that mRNA levels of COX-1 and fibronectin decreased remarkably to the minimum level by phenol treatment, whereas G3PDH expression increased compared to mRNA levels of the intact skin. When wogonin was applied to the phenol-treated skin, fibronectin gene expression increased slightly at 50 μg/ear. IL-1β expression level was weakly suppressed at 200 μg/ear (23.1% reduction), while prednisolone (50 μg/ear) strongly reduced IL-1β expression level (75.0% reduction). The expression levels of other genes were not significantly changed by topical application of wogonin and prednisolone, compared with those of the phenol-treated group.

In the delayed-type contact dermatitis model, picryl chloride treatment to the sensitized mice (the elicitation phase reaction, P-P) increased ear thickness to 0.32±0.01 mm, while the induction phase reaction with picryl chloride (A-P) gave 0.26±0.02 mm ear thickness in 24 h compared

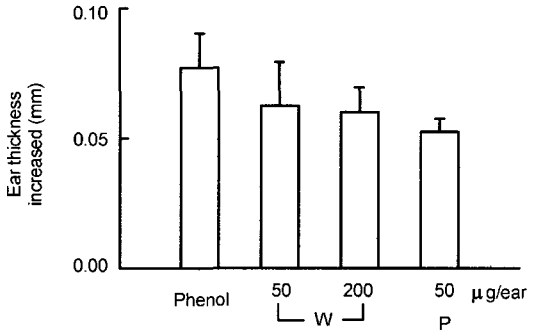

Fig. 2. Inhibition of wogonin against phenol-induced ear edema. Phenol (10%) was applied and, two hours later, ear thickness was measured as described in Materials and Methods. Each value means arithmetic mean ± SD (n = 5). No statistical significance was observed in wogonin (W)- and prednisolone (P)-treated groups compared to the phenol-treated group.

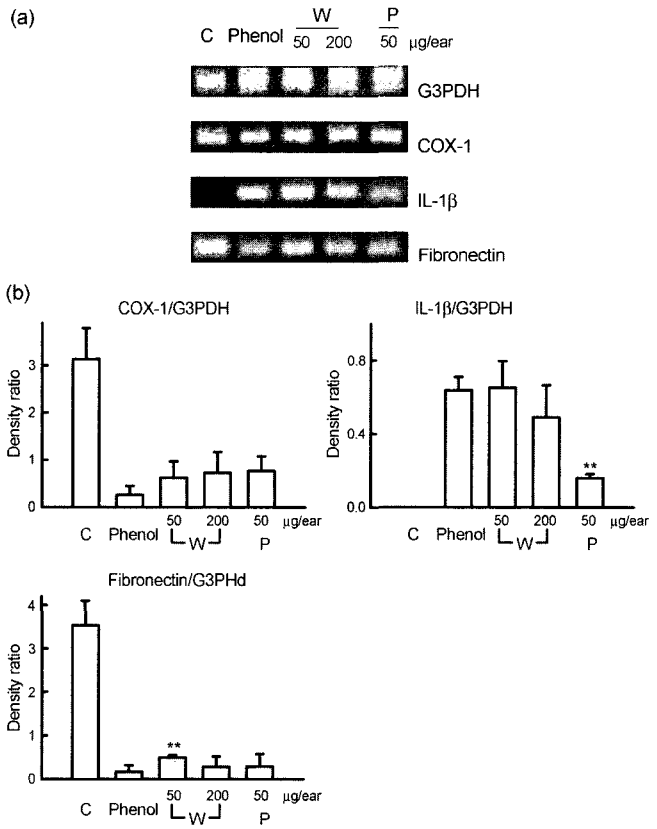

Fig. 3. Effects of wogonin on proinflammatory gene expression of the phenol-treated skin. (a) RT-PCR analysis. One representative PCR result among three analyses from each animal group is shown here. (b) Relative band density ratio. The ratio of band density compared to G3PDH is represented as arithmetic mean ± SD (n = 3). **: P < 0.05, significantly different from the phenol-treated group. Vehicle-treated control (C), wogonin (W), prednisolone (P).

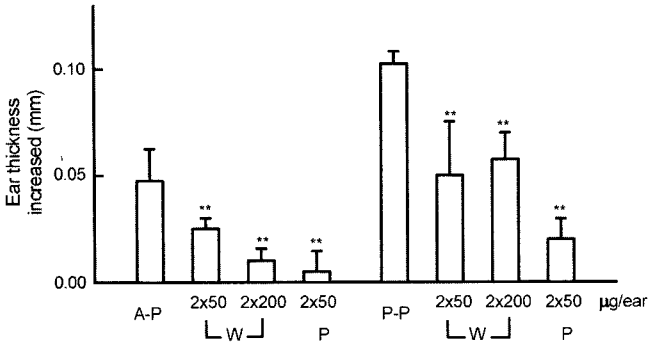

Fig. 4. Inhibition of wogonin against ear edema by picryl chloride-induced delayed hypersensitivity. Ear thickness was measured 24 h after final treatment with picryl chloride/acetone. A-P represents the induction phase reaction by picryl chloride treatment to the unsensitized mice and P-P represents the elicitation phase reaction of delayed hypersensitivity. Each value means arithmetic mean ± SD (n = 5). **: P < 0.05, significantly different from A-P or P-P group, respectively (n = 5). Wogonin (W), prednisolone (P).

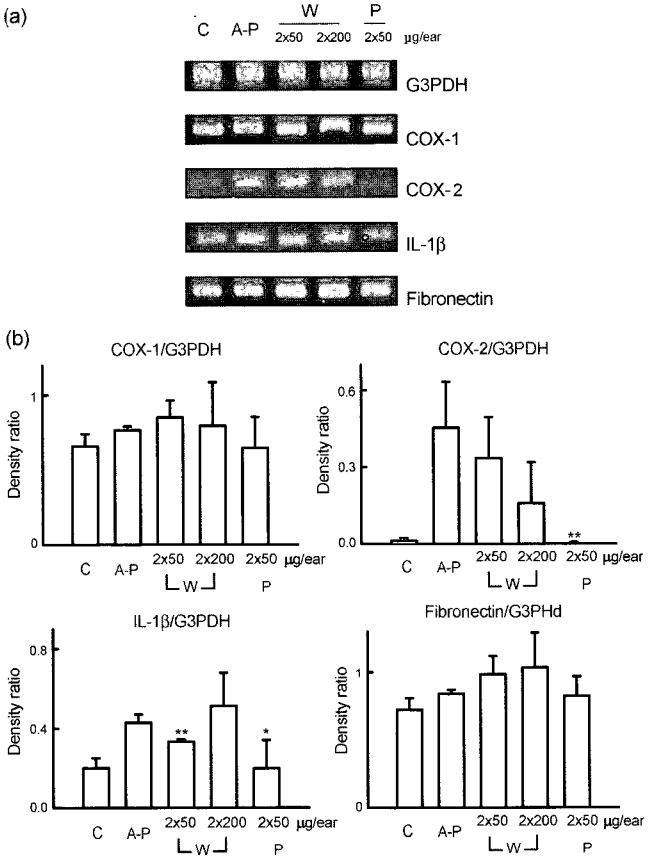

Fig. 5. Effects of wogonin on proinflammatory gene expression of the induction phase reaction by picryl chloride treatment (A-P). (a) RT-PCR analysis. One representative PCR result among three analyses from each animal group is shown here. (b) Relative band density ratio. The ratio of band density compared to G3PDH is represented as arithmetic mean ± SD (n = 3). *: P < 0.1, **: P < 0.05, significantly different from the phenol-treated group. Vehicle-treated control (C), wogonin (W), prednisolone (P).

with the control group of 0.21±0.01 mm (n = 5). Under this condition, wogonin significantly inhibited ear edema of the induction phase reaction (A-P) in a dose-dependent manner, showing 47.4% and 79.0% inhibition at 2×50 and 2×200 μg/ear, respectively (Fig. 4). Prednisolone inhibited ear edema against the induction phase reaction by 89.5% at 2×50 μg/ear. In the induction phase reaction with picryl chloride treatment to the unsensitized mice (A-P), COX-2 gene was considerably induced, while mRNA of IL-1β was slightly induced (Fig. 5). Other inducible genes checked were not detected. In contrast with phenol treatment, the levels of the constitutive genes such as G3PDH, COX-1 and fibronectin were constantly expressed as expected. When wogonin was applied, COX-2 expression decreased dose-dependently (26.1% and 65.3% reduction at 2×50 and 2×200 μg/ear, respectively), whereas IL-1β gene expression was reduced only slightly at 2×50 μg/ear (31.8% reduction). Other gene levels were not significantly changed, despite the slight increase of COX-1 and fibronectin gene levels. Prednisolone (2×50 μg/ear) showed similar patterns of gene expression, but showed stronger reduction of COX-2 expression (99.3% reduction) and IL-1β expression (59.6% reduction) than those of wogonin.

In the elicitation phase of the delayed hypersensitivity reaction (P-P), wogonin moderately inhibited edematic response by 51.2% and 43.9% reduction at 2×50 and 2×200 μg/ear, respectively, while prednisolone potently reduced ear edema by 80.5% (Fig. 4). When RT-PCR analysis was carried out, COX-2, IL-1β and IFN-γ genes were significantly up-regulated in the elicitation phase reaction (P-P) (Fig. 6). ICAM-1 gene was weakly induced, and iNOS mRNA was weakly detected from two out of three ear samples. The constitutive genes were constantly detected. When topically applied, wogonin strongly inhibited COX-2 (66.0% and 65.0%), ICAM-1 (99.6% and 71.1%) and iNOS induction (91.4% and 45.8% reduction) at 2×50 and 2×200 μg/ear, respectively, being more potent at 2×50 μg/ear. These results were well correlated with the inhibition of ear edema by wogonin showing higher inhibition at low dose treatment. As a comparison, wogonin weakly suppressed IL-1β (42.2% and 18.2% reduction) and IFN-γ expression (57.3% and 25.0% reduction) at 2×50 and 2× 200 μg/ear, respectively. Again, low-dose wogonin treatment showed higher inhibition. When the expression levels of the constitutive genes were compared, unexpected results were obtained; low-dose treatment with wogonin (2×50 μg/ear) showed a weak reduction of the expression of COX-1 and fibronectin genes (24.7% and 21.0% reduction, respectively). Prednisolone showed inhibitory patterns against proinflammatory gene expression that were similar to wogonin. The inhibition degrees of COX-2, IL-1β,

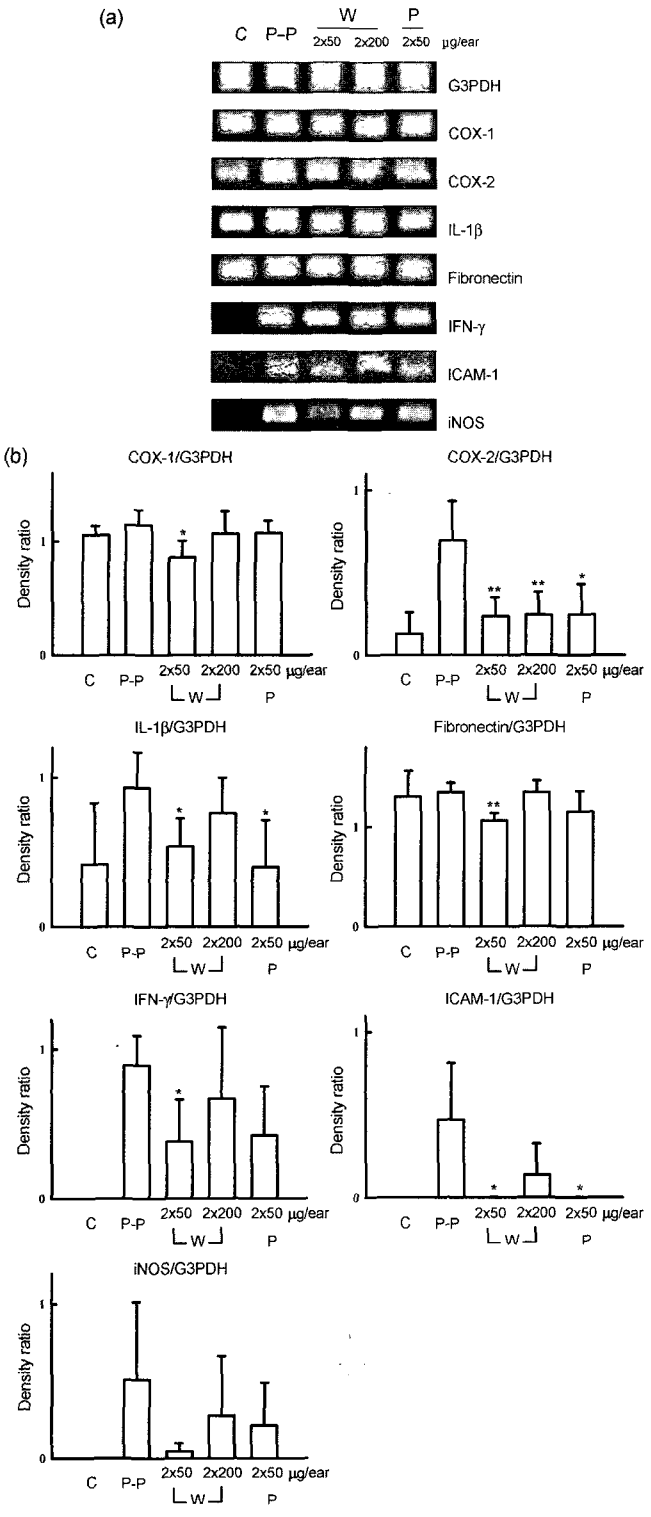

Fig. 6. Effects of wogonin on proinflammatory gene expression of the elicitation phase reaction of delayed hypersensitivity (P-P). (a) RT-PCR analysis. One representative PCR result among three analyses from each animal group is shown here. (b) Relative band density ratio. The ratio of band density compared to G3PDH is represented as arithmetic mean ± SD (n = 3). *: P < 0.1, **: P < 0.05, significantly different from the phenol-treated group. Vehicle-treated control (C), wogonin (W), prednisolone (P).

ICAM-1, IFN-γ and iNOS induction by prednisolone were 64.5%, 57.0%, 99.6%, 52.6% and 58.3%, respectively.

DISCUSSION

The present study clearly demonstrated that the topical application of wogonin inhibited the skin inflammation of contact dermatitis: simple irritation and both the induction phase and the elicitation phase of delayed hypersensitivity on animal skin. Wogonin not only inhibited the edematic response, but also down-regulated several types of pro-inflammatory gene expression.

Depending on the animal models of contact dermatitis and the end-point time used to measure the response, there were considerable differences in the nature and patterns of proinflammatory gene expression. For example, multiple TPA treatment in mice for three consecutive days led to the induction of proinflammatory genes such as COX-2, IL-1β, TNF-α and ICAM-1 (Chi *et al.*, 2003). A single TPA treatment on mouse skin was also observed to induce the expression of COX-2, TNF-α and transforming growth factor (TGF)-β1 in 4 h (Jang and Pezzuto, 1998). In the present study, phenol treatment only induced IL-1β gene expression after 2 h, among the inducible genes tested. On the other hand, COX-2 and IL-1β genes were up-regulated in the induction phase of picryl chloride-induced dermatitis (A-P). In the elicitation phase of the delayed hypersensitivity reaction (P-P), COX-2, IL-1β, IFN-γ, ICAM-1, and iNOS genes were detected 24 h after the last treatment with the sensitizer. Among proinflammatory molecules, IL-1β was previously found to be an important factor in inducing a delayed-type hypersensitivity reaction (Shornick *et al.*, 1996). Adhesion molecules including ICAM-1, vascular cell adhesion molecule (VCAM)-1 and endothelial leukocyte adhesion molecule (ELAM)-1 were also significantly involved in the same reaction (Norris *et al.*, 1991). In our experiment of delayed hypersensitivity, IL-1β and ICAM-1 mRNAs were detected. Although the TNF-α gene was not detected by RT-PCR analysis, this gene was found to be critically involved in a similar hypersensitivity reaction (Piguet *et al.*, 1991; Mitsui *et al.*, 2003). It is not known at present why we could not detect TNF-α mRNA, but it may be due to different sensitizers used, different treatment protocols and different mouse strains employed. The most striking difference of expression patterns in animal models of contact dermatitis is related to IFN-γ and iNOS expression. These molecules are up-regulated in the elicitation phase of the delayed hypersensitivity reaction. In contrast, no band of IFN-γ and iNOS was found in phenol-induced simple irritation.

Wogonin was previously found to be a down-regulator of COX-2 and iNOS induction from macrophages in culture (Chi *et al.*, 2001). It was also reported that

wogonin inhibited several parameters including edema and the proinflammatory gene expression of sub-chronic skin inflammation provoked by multiple TPA treatment in mice (Park *et al.*, 2001; Chi *et al.*, 2003). This skin inflammation model is characterized by elevated levels of COX-2 and several other proinflammatory molecules as noted above. The infiltration of large numbers of neutrophils/monocytes is another aspect of TPA-induced inflammatory response, and prolonged exposure to TPA possibly leads to carcinogenesis.

Phenol-induced dermatitis is one of the animal models of contact dermatitis, and is used as a model to produce immediate irritation. Phenol produced massive edema in 2 h. The fact that wogonin weakly reduced the edematic response of phenol-induced dermatitis suggests the edema produced by this model may be quite different from that of sub-chronic skin inflammation caused by TPA over 3 days as described above. However, at present, it is not understood why phenol treatment drastically reduced mRNA levels of the constitutive genes, COX-1 and fibronectin, while G3PDH expression increased considerably.

Picryl chloride-induced delayed hypersensitivity, on the other hand, is an allergic contact dermatitis. It is essentially a cell-mediated immune response composed of the induction phase reaction by first contact with the allergen and the elicitation phase reaction by re-exposure to the same allergen (Willis *et al.*, 1986; Rietschel and Fowler, 1995). In this animal model, wogonin potently inhibited the induction phase reaction of contact dermatitis (A-P), but was less potent against the elicitation phase reaction of delayed sensitized hypersensitivity (P-P). Although the precise reason is not known, the different sensitivity of wogonin may be partly explained by the intrinsic differences of the induction phase (A-P) and the elicitation phase (P-P) reactions including the different inflammatory cells and cytokines involved in each reaction. To the sensitized site of the elicitation phase in picryl chloride-induced hypersensitivity reaction (P-P), lymphocytes are infiltrated and especially T-cells are known to play a major role (Mitsui *et al.*, 2003). Therefore, it is speculated that wogonin may not strongly affect the functions of lymphocytes. In a recent report, wogonin was described as inhibiting IgE production from T-cell mitogen-induced splenocytes and histamine release from peritoneal cells (Lim *et al.*, 2003). Although this finding indicates that wogonin may reduce the type 1 hypersensitivity reaction, effects on T-cells and their proinflammatory gene expression remain to be elucidated. In contrast, prednisolone showed potent inhibition of both the induction and the elicitation phases of delayed hypersensitivity as expected.

From the present study, the patterns of down-regulating proinflammatory gene expression by wogonin and prednisolone were revealed to be very similar, despite some different sensitivities. However, the cellular action mechanisms down-regulating proinflammatory gene expression by these agents are quite different. Our previous study showed that prednisolone lost its suppressive activity on iNOS induction from LPS-induced RAW 264.7 cells by the addition of a steroid receptor antagonist (Ru-486), but wogonin did not (Chi *et al.*, 2001). This finding and others (Goppelt-Struebe, 1997) indicate that prednisolone suppresses proinflammatory gene expression by the inhibition of transcription factor activation partly *via* a steroid-receptor mediated process. On the other hand, flavonoids including wogonin, luteolin and quercetin may reduce proinflammatory gene expression by the inhibition of transcription factor activation probably *via* the inhibition of protein kinases involved in the signal transduction pathway (Chang *et al.*, 2001; Xagorari *et al.*, 2002; Wadsworth *et al.*, 2001). Thus, it is expected that wogonin may not show steroid-like side effects in human use.

In conclusion, wogonin showed anti-inflammatory activity against animal models of contact dermatitis: simple irritation and delayed hypersensitivity. Although the nature and patterns of proinflammatory gene expression were different in each animal models of contact dermatitis used, wogonin generally down-regulated the COX-2 gene strongly, with less of an effect on IFN-γ and IL-1β induction *in vivo*. And this down-regulating property of several proinflammatory genes by wogonin may, at least in part, contribute to its anti-edematic and anti-inflammatory responses against animal models of contact dermatitis. Wogonin may have potential as a new anti-inflammatory agent against skin inflammatory disorders.

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