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Original Contribution

PINE BARK EXTRACT PYCNOGENOL DOWNREGULATES IFN-γ-INDUCED ADHESION OF T CELLS TO HUMAN KERATINOCYTES BY INHIBITING INDUCIBLE ICAM-1 EXPRESSION

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Abstract—Expression of intercellular adhesion molecule-1 (ICAM-1) is necessary for leukocyte/keratinocyte interactions. Upregulation of ICAM-1 expression in keratinocytes has been observed in several inflammatory dermatoses, such as psoriasis, atopic dermatitis, and lupus erythematosus. Inflammatory cytokines, such as interferon-γ (IFN-γ), upregulate ICAM-1 expression in keratinocytes. Because of potent antioxidant and anti-inflammatory properties of the French maritime pine bark extract, Pycnogenol (Horphag Research, Geneva, Switzerland), its effects were investigated on the interaction of T cells with keratinocytes after activation with IFN-γ and the molecular mechanisms involved in such interactions. Studies were performed using a human keratinocyte cell line, HaCaT. Cell adhesion in the presence of IFN-γ was studied using a coculture assay. Treatment of HaCaT cells with 20 U/ml IFN-γ for 24 h markedly induced adherence of Jurkat T cells to HaCaT cells. PYC pretreatment (50 μg/ml, 12 h) significantly inhibited IFN-γ induced adherence of T cells to HaCaT cells ($p < .01$). ICAM-1 plays a major role in the IFN-γ-induced adherence of T cells to keratinocytes. Thus, the effect of PYC on IFN-γ-induced ICAM-1 expression was investigated as well. Pretreatment of HaCaT cells with PYC significantly inhibited IFN-γ-induced expression of ICAM-1 expression in HaCaT cells. The downregulation of inducible ICAM-1 expression by PYC was both dose and time dependent. A 50 μg/ml dose of PYC and a 12 h pretreatment time (i.e., before activation with IFN-γ) provided maximal (~70%) inhibition of inducible ICAM-1 expression in HaCaT cells. Gamma-activated sequence present on the ICAM-1 gene confers IFN-γ responsiveness in selected cells of epithelial origin (e.g., keratinocytes) that are known to express ICAM-1 on activation with IFN-γ. Gel-shift assays revealed that PYC inhibits IFN-γ-mediated activation of Stat1, thus suggesting a transcriptional regulation of inducible ICAM-1 expression by PYC. These results indicate the therapeutic potential of PYC in patients with inflammatory skin disorders.

Keywords—Flavonoids, Procyanidins, Plant polyphenols, Reactive oxygen species, Skin inflammation, Stat1, Free radicals

INTRODUCTION

As a protective interface between internal organs and the environment, the skin encounters a host of pathogenic organisms as well as physical and environmental stressors, such as toxic chemicals, UV rays, and ozone. To defend against such challenges to the cutaneous microenvironment, the skin functions as more than just a physical barrier; it is also an active immune organ. Upregulation of the expression of ICAM-1, which is an immunoglobulinlike, 90 kDa glycoprotein, in keratinocytes is an important feature of cutaneous inflammation [1,2]. Increased expression of ICAM-1 has been demonstrated in established inflammatory dermatoses [3], experimental delayed hypersensitivity [4], and other cutaneous inflammatory conditions, such as psoriasis, atopic dermatitis, and lupus erythematosus [5]. T lymphocytes play an important role in the generation, maintenance, and specificity of the skin immune response. In addition, the primary class of cutaneous lymphomas is believed to be of T-cell lineage [6]. LFA-1 is a major surface glycoprotein on T cells, serves as a ligand for ICAM-1 and

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promotes intercellular adhesion in immunologic and inflammatory reactions. IFN-γ, which is a pleiotropic cytokine produced by activated T lymphocytes, plays a critical role in host defenses and inflammation [7]. In the skin, IFN-γ induces de novo expression of ICAM-1 on human keratinocytes, which are critically involved in cutaneous inflammatory processes [8].

Because of its central role in cutaneous inflammation, controlling the inducible expression of ICAM-1 and the interaction of ICAM-1 (i.e., keratinocytes) with LFA-1 (i.e., T cells) has been an important therapeutic target. Only a few agents, such as corticosteroids, antihistamines, or immunosuppressants, are available that are known to be effective in the treatment of skin inflammation. All these agents, however, have limitations. For example, antihistamines are not as potent as other agents, and they may be used merely as supplemental therapy [9,10]. Corticosteroids and immunosuppressants have potent inhibitory effects but have problems as long-term continuous medication [11,12]. Thus, long-term and safe control of inflammation remains a challenge in the field of therapy for skin inflammation.

Pycnogenol (PYC) (Horphag Research, Geneva, Switzerland), which is an extract prepared from the bark of the French maritime pine *(Pinus maritima)*, is a unique complex of flavonoids, mainly procyanidins, phenolic acids, and other components [13]. Previous results have indicated that after a single 200-mg dose of PYC supplementation to human, components (e.g., ferulic acid) of PYC appear in urine [13]. Several studies have elucidated antioxidant as well as anti-inflammatory properties of this extract [13], and plant extract from tea or ginkgo has also been reported to have various clinically relevant properties, such as antioxidant [14–17] and anti-inflammatory activity [18,19]. We previously showed that bioflavonoids such as quercetin are potent inhibitors of inducible ICAM-1 expression [20]. In the present study, the efficacy of PYC in regulating IFN-γ-induced adherence of keratinocytes with T cells and the molecular mechanisms involved in such interactions were investigated. To gain insight regarding which component of PYC is effective, the effects of various constituent flavonoids (i.e., catechin, ferulic acid, and taxifolin) on inducible ICAM-1 expression and T-cell adhesion to keratinocytes were also investigated.

MATERIALS AND METHODS

Cells and cell culture

The immortalized human keratinocytes cell line HaCaT (used with the permission of Dr. N. E. Fusenig, Heidelberg, Germany) was cultured using Dulbecco's modified Eagle's medium (Life Technologies, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (University of California, San Francisco, CA, USA). Human Jurkat T cells clone E6-1 (American Type Culture Collection; ATCC, Bethesda, MD, USA) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 110 mg/L sodium pyruvate, and 2 mM L-glutamine (University of California). Cells were maintained in a standard culture incubator with humidified air containing 5% CO_2 at 37°C.

The PYC and other flavonoid (Sigma Chemical, St. Louis, MO, USA) stock solutions were prepared fresh in DMSO so that the final concentration of the solvent in cell suspension never exceeded 0.1% (v/v). Respective controls were treated with an equal volume of DMSO.

Adhesion assay

The effect of PYC and constituent flavonoids on the inducible adherence of Jurkat T cells to HaCaT cells was assayed using a cell–cell adhesion assay as described elsewhere [20–22].

Calcein labeling of cells. Jurkat T cells (1×10^7 cells/ml) were fluorescent labeled by incubation with 5 μM calcein acetoxymethyl ester (calcein-AM; Molecular Probes, Eugene, OR, USA) in PBS at a pH of 7.4 for 30 min at 37°C. After loading of calcein-AM, cells were washed three times with PBS to remove excess dye. Cells were then resuspended in phenol red–free RPMI 1640 containing 10% fetal bovine serum at a density of 3×10^6 cells/ml.

Cell–cell adhesion assay. Monolayers of HaCaT cells were seeded at a density of 1×10^4 cells/well in 96 well tissue-culture plates (Falcon 3072; Becton Dickinson, Franklin Lakes, NJ, USA). After 24 h of seeding, cells were treated with PYC or flavonoids for the time periods indicated in respective figure legends. After the treatment period, cells were activated with 20 U/ml IFN-γ (Genzyme, Cambridge, MA, USA) for 24 h. The HaCaT monolayers were washed three times with PBS before cell–cell adhesion assay. Calcein-AM-labeled Jurkat T cells (3×10^5 cells/well) were cocultured with HaCaT monolayers for 1 h in a culture incubator with humidified air containing 5% CO_2 at 37°C. After the coculture period, the nonadherent Jurkat T cells were removed from the monolayers by washing each well four times with PBS using a multichannel pipette with wide, open tips. The fluorescence intensity of each well was measured using a fluorescence plate reader (CS-9301; Shimadzu, Columbia, MD, USA). The excitation and emis-

sion wavelengths for the calcein molecule are 480 and 530 nm, respectively. The relative contributions of ICAM-1 to the adherence of Jurkat T cells to HaCaT cells was evaluated by treating HaCaT cells with anti-human ICAM-1 (10 μg/ml) antibody (Beckman Coulter, Fullerton, CA, USA) for 30 min before coculture of HaCaT cells with Jurkat T cells.

Expression of ICAM-1

HaCaT monolayers were pretreated with PYC or flavonoids for the time period indicated in the respective figure legends. After pretreatment, cells were activated using 20 U/ml IFN-γ for 24 h. To study ICAM-1 expression, cells were incubated using FITC coupled to ICAM-1 monoclonal antibody (Immunotech, Cedex, France) for 1 h at 4°C. Expression of ICAM-1 was immediately assayed using a flow cytometer. Cells with FITC-conjugated antibodies were excited using a 488 nm argon-ion laser, and emission of FTIC was recorded at 525 nm. In each sample, at least 10,000 gated, viable cells were examined. Appropriate isotypic controls were used for background fluorescence in the ICAM-1 assay [22].

RNA isolation and Northern blot analysis

HaCaT cells were grown to confluence in 10 cm cell culture dishes. Total RNA was extracted from HaCaT cells using the method of Chomczynski and Sacchi [23].

The RNA samples (10 μg) were subjected to electrophoresis in 1% (w/v) formaldehyde-agarose gels and transferred to Hybond-N nylon membranes (Amersham, Piscataway, NJ, USA) overnight in 10× SSC (1.5 M NaCl; 0.15 M sodium citrate; pH, 7.0). RNA was cross-linked to the nylon membrane using a 5 min UV exposure. Blots were then prehybridized for at least 1 h at 37°C in 50% formamide, 5× SSC, 0.1% SDS, 5× Denhardt's solution (0.1% bovine serum albumin, Ficoll, and polyvinylpyrrolidine), and 100 μg/ml denatured sperm DNA (Life Technology, Gaithersburg, MD, USA). Blots were hybridized at 37°C for 16 h using a human [$^{\gamma\text{-}32}$P]-labeled ICAM-1 cDNA probe. cDNA probes were labeled with [$^{\gamma\text{-}32}$P]-ATP using T4 polynucleotide kinase (Boehringer Mannheim, Indianapolis, IN, USA). Blots were washed twice with wash I solution (1× SSC, 0.1% SDS) for 10 min at 3°C, which was followed by two more washes in wash II solution (0.2× SSC, 0.1% SDS) for 10 min at 60°C. To normalize the mRNA content, blots were stripped and reprobed with a radiolabeled human β-actin cDNA probe [20].

Electrophoretic mobility shift assay

HaCaT cells were preincubated using PYC or flavonoids and then treated with IFN-γ. After appropriate activation periods (as indicated in the respective figure legends), the nuclear extracts were prepared and EMSAs performed essentially as described earlier [22]. Binding reaction mixtures (20 μl) containing 5 μg protein of nuclear extract, 2 μg poly (dI-dC) (Pharmacia, Sweden), ^{32}P-labeled probe (Stat1 or IRF-1), 50 mM NaCl, 2 mM $MgCl_2$, 0.2 mM Na_2EDTA, 1 mM DTT, 10 % (v/v) glycerol, and 4 mM Tris-HCl (pH, 7.9) were incubated for 30 min at room temperature. Proteins were separated using electrophoresis in a native 6% polyacrylamide gel using a Tris-borate-EDTA running buffer (12.5 mM Tris-borate containing 0.25 mM Na_2EDTA; pH, 8.0) followed by autoradiography. The Stat1 probe (Santa Cruz, CA, USA), or 5′. . .CAT GTT ATG CAT ATT CCT GTA AGT G. . .3′; 3′. . .GTA CAA TAG GTA TAA GGA CAT TCA C. . .5′, and the IRF-1 probe (Santa Cruz, CA, USA), or 5′. . .GGA AGC GAA AAT GAA ATT GAC T. . .3′; 3′. . .CCT TCG CTT TTA CTT TAA CTG A. . .5′, were labeled with [$^{\gamma\text{-}32}$P] dATP (Du Pont NEN, Boston, MA, USA) using T4 polynucleotide kinase.

Statistical analysis

Data are presented as mean ± SD of at least three separate experiments. Differences between the means of groups were analyzed using one-way analysis of variance and Student's *t*-test, with $p < .05$ considered to be significant.

RESULTS

Adhesion of Jurkat T lymphocytes to IFN-γ-treated HaCaT cells

The coculture assay was used to evaluate the efficacy of PYC and constituent flavonoids for regulation of IFN-γ-induced cell–cell adherence. Pretreatment of HaCaT cells with PYC dose dependently (5–50 μg/ml) inhibited IFN-γ-induced adherence of Jurkat T cells to HaCaT cells (Fig. 1). Approximately 60% inhibition of inducible cell–cell adhesion was noted at the PRC dose of 50 μg/ml after 12 h of pretreatment. Treatment of HaCaT cells with anti-ICAM-1–blocking antibody markedly (~70%) inhibited IFN-γ-induced Jurkat T-cell adhesion to HaCaT cells (Fig. 1).

Using equal concentrations (30 μg/ml) of PYC and its constituent flavonoids (i.e., catechin, ferulic acid, taxifolin), the effect on IFN-γ-induced adherence of T cells to HaCaT cells was determined (Fig. 2). The 30 μg/ml dose

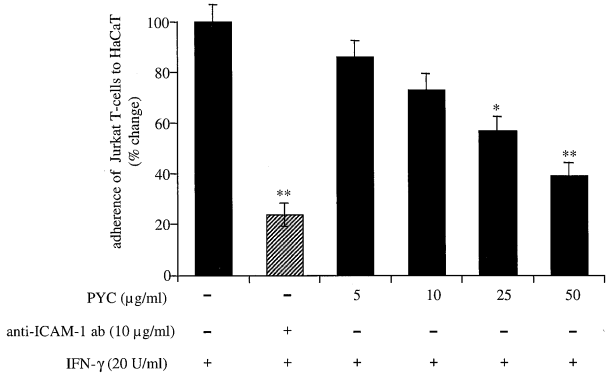

Fig. 1. Adhesion of human Jurkat T cells to IFN-γ-activated HaCaT cells is inhibited by PYC pretreatment. HaCaT cells were seeded at a density of 1×10^4 cells/well in 96-well tissue-culture plates. After 24 h of seeding, cells were treated with PYC (5–50 μg/ml) for 12 h and then activated with 20-U/ml IFN-γ for 24 h. Cells were washed three times with PBS and then cocultured with calcein-AM-labeled Jurkat T cells for 1 h. The fluorescence from the adherent Jurkat T cells was measured using a multiple-well plate leader. The relative contribution of ICAM-1 to inducible cell–cell adhesion was determined by incubating HaCaT cells with ICAM-1-blocking antibody (ab; 10 μg/ml) for 30 min before the assay. Data are the mean ± SD of at least three experiments. *$p <$ .05 and **$p <$ .01 compared with non-PYC treated cells.

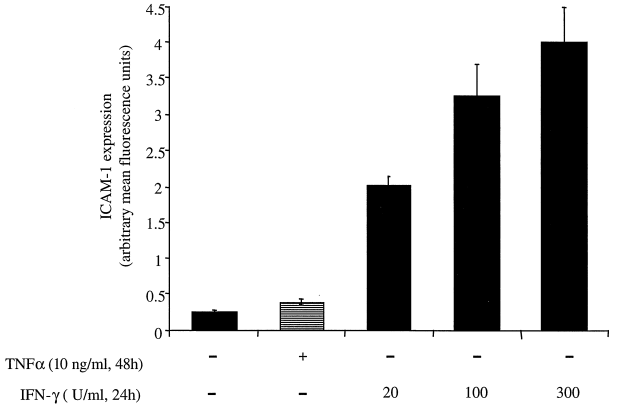

Fig. 3. Dose relationship of IFN-γ and TNF-α activation on ICAM-1 expression in HaCaT cells. Cells were treated with IFN-γ (20–300 U/ml for 24 h) or TNF-α (10 ng/ml for 48 h). ICAM-1 expression was determined using a flow cytometer as described in the text. Data are the mean ± SD of at least three experiments.

was selected because, at greater doses, taxifolin has cytotoxic effects in HaCaT cells (data not shown). At the concentrations tested, all flavonoids and PYC showed downregulation in the inducible cell–cell adhesion. The percent changes, however, varied from least (~20%) for catechin to a maximum (~50%) for PYC (Fig. 2).

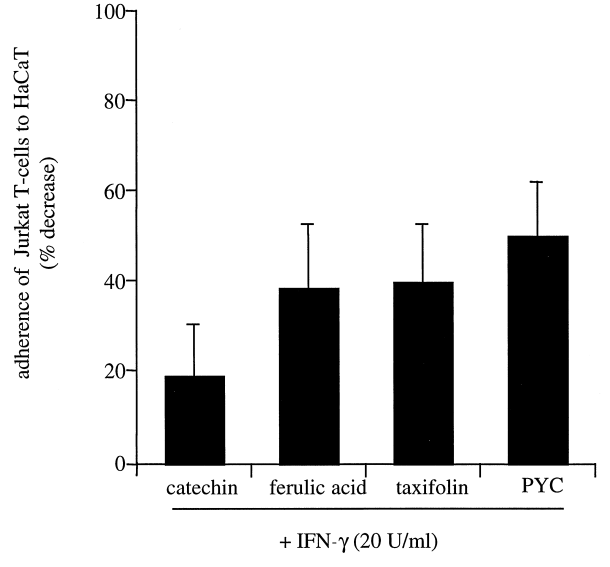

Fig. 2. PYC and its constituent flavonoids inhibit adherence of Jurkat T cells to IFN-γ-activated HaCaT cells. HaCaT cells were pretreated with equal concentrations (30 μg/ml) of catechin, ferulic acid, taxifolin, or PYC for 12 h, which was followed by activation with IFN-γ (20 U/ml) for 24 h. The cell–cell adhesion assay was performed as described in Fig. 1. Data are presented as the percent decrease compared with nonpretreated and IFN-γ-activated cells. Data are the mean ± SD of at least three experiments.

ICAM-1 expression

The effect of PYC and constituent flavonoids on IFN-γ-induced ICAM-1 expression in HaCaT cells was also investigated. Basal ICAM-1 expression levels were very low in unstimulated cells. Treatment of cells with IFN-γ for 24 h dose dependently (20–300 U/ml) increased the expression of ICAM-1 (Fig. 3). In subsequent experiments, a 20 U/ml dose of IFN-γ was used. Treatment with TNF-α, which is a potent inducer of ICAM-1 in endothelial cells and some keratinocytes, for 24 (data not shown) or 48 h did not affect the ICAM-1 expression in HaCaT cells (Fig. 3).

Treatment of cells with PYC or flavonoids alone had no effect on the basal expression of ICAM-1 by HaCaT cells (data not shown). A marked inhibition in inducible ICAM-1 expression was observed in cells that were treated with PYC (50 μg/ml) for 12 h (Fig. 4). Such downregulation of IFN-γ-induced ICAM-1 expression by PYC was dose dependent (5–50 μg/ml). The effect was already significant (~30 %, $p <$ .05) in cells that were pretreated with 25 μg/ml PYC compared with IFN-γ-activated, nontreated cells (Fig. 5).

To characterize the kinetics of the inhibitory effect of PYC on IFN-γ-induced expression of ICAM-1, HaCaT cells were pretreated with 50 μg/ml of PYC for different time periods (1–15 h) and then activated with IFN-γ for 24 h (Fig. 6). A significant decrease in the IFN-γ-induced levels of ICAM-1 was observed in cells at as early as 6 h of pretreatment with 50 μg/ml PYC ($p <$ .01). Maximal inhibition of inducible ICAM-1 expression was

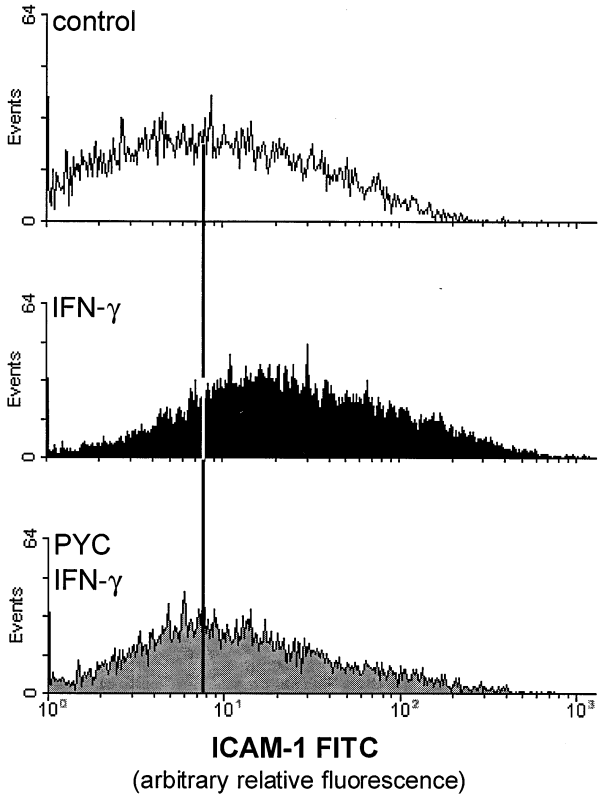

Fig. 4. PYC downregulates IFN-γ-induced ICAM-1 expression in HaCaT cells. Flow cytometric analysis was performed on HaCaT cells pretreated with PYC (50 μg/ml for 12 h) followed by activation with 20 U/ml IFN-γ for 24 h. The solid line represents the median value of the log ICAM-1 FITC signal of control (no treatment) cells. Data are presented as histograms of cell number versus log ICAM-1 FITC fluorescence intensity. Histograms represent one of the three essentially identical experiments.

observed in cells that were pretreated with PYC for 12 h (Fig. 6).

The effects of equal concentrations (30 μg/ml) of PYC and its constituent flavonoids on IFN-γ-induced ICAM-1 expression in HaCaT cells was compared as well (Fig. 7). A significant decrease in expression was observed among cells that were pretreated with taxifolin (~15% inhibition) or PYC (~35% inhibition). Catechin or ferulic acid had no significant effect on inducible ICAM-1 expression (Fig. 7). The downregulatory effects of these substances on inducible ICAM-1 expression were not the result of cytotoxicity, because no significant differences in viability were observed between non-treated or flavonoid/PYC-treated cells as determined using the lactate dehydrogenase leakage test (data not shown).

ICAM-1 mRNA expression

To characterize the molecular mechanisms responsible for the downregulation of IFN-γ-induced ICAM-1 expression by PYC, the effect on the ICAM-1 mRNA level was investigated using Northern blot analysis. HaCaT cells were pretreated with 50 μg/ml PYC for 12 h and then activated using 20 U/ml IFN-γ for 4 h. HaCaT cells exhibited very low levels of ICAM-1 mRNA in the nonactivated cells, but IFN-γ (20 U/ml, 4 h) markedly increased ICAM-1 mRNA levels in HaCaT cells. PYC pretreatment of 50 μg/ml for 12 h almost completely suppressed IFN-γ-induced ICAM-1 mRNA accumulation (Fig. 8).

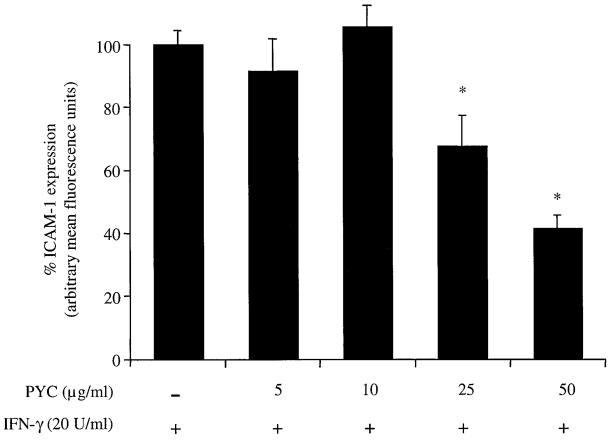

Fig. 5. Dose relationship of PYC pretreatment and IFN-γ-induced ICAM-1 expression in HaCaT cells. Cells were pretreated with PYC (5–50 μg/ml) for 12 h, which was followed by activation with IFN-γ (20 U/ml) for 24 h. ICAM-1 expression was determined using a flow cytometer as described in the text. Data are the mean ± SD of at least three experiments. *$p < .01$ compared with non-PYC treated cells.

Transcriptional regulation of IFN-γ-induced ICAM-1 mRNA

The binding motif of GAS has been identified in the ICAM-1 promoter, and binding of Stat1 to this motif

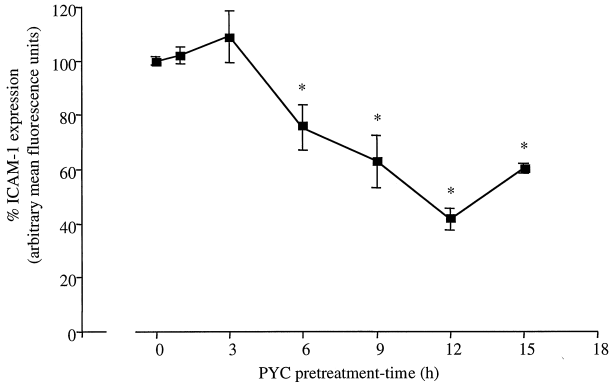

Fig. 6. Effect of PYC pretreatment time on IFN-γ-induced ICAM-1 expression. HaCaT cells were pretreated with PYC (50 μg/ml) for different time periods (1–15 h) and then activated with IFN-γ (20 U/ml) for 24 h. Data are the mean ± SD of at least three experiments. *$p < .01$ compared with non-PYC treated cells.

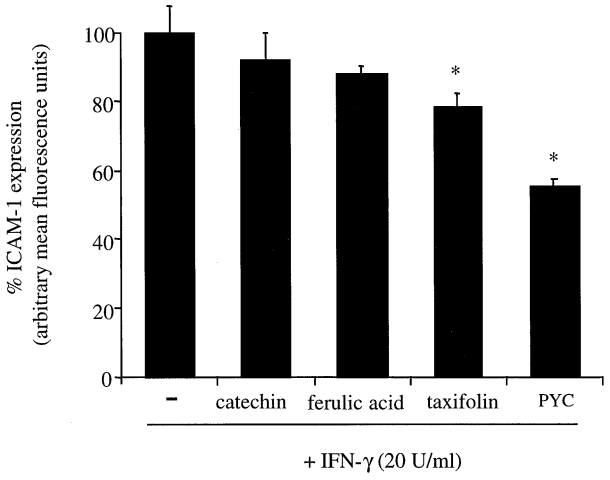

Fig. 7. Effect of PYC and its constituent flavonoids on IFN-γ-induced ICAM-1 expression. HaCaT cells were pretreated with equal concentrations (30 μg/ml) of catechin, ferulic acid, taxifolin, or PYC for 12 h and then activated with IFN-γ (20 U/ml) for 24 h. Data are the mean ± SD of at least three experiments. *$p < .01$ compared with non-PYC treated cells.

plays a major role in transcriptional activation of the IFN-γ-induced ICAM-1 gene. The PYC effect on the activation of Stat1 was investigated in this study using EMSA. HaCaT cells were treated with 20 U/ml IFN-γ for various time periods. Maximum activation of Stat1 was observed after 30 min of IFN-γ treatment (Fig. 9A). The specificity of the Stat1 signal was confirmed by cold competition using excess unlabeled Stat1 probe. Use of excess unlabelled probe decreased the Stat1-specific signal. A dose-dependent (10–50 μg/ml) decrease in the IFN-γ-mediated activation of Stat1 was observed in HaCaT cells that were pretreated with PYC for 12 h (Fig. 9B). Marginal inhibition of IFN-γ-induced Stat1 activation was observed in cells that were pretreated with taxifolin (30 μg/ml) for 12 h (data not shown). Pretreatment of HaCaT cells with catechin or ferulic acid up to

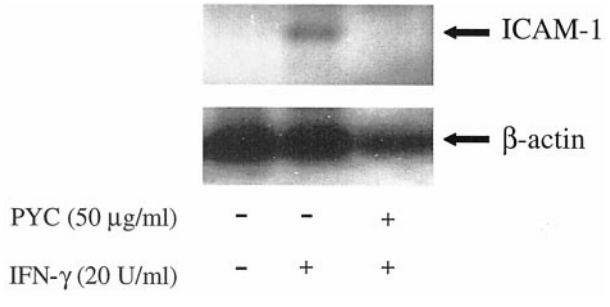

Fig. 8. Effect of PYC on IFN-γ-induced ICAM-1 mRNA expression. HaCaT cells were pretreated with PYC (50 μg/ml) for 12 h and then activated with IFN-γ (20 U/ml) for 4 h. Levels of mRNA were determined by Northern blot analysis using ICAM-1 or β-actin (reference) probes.

a dose of 50 μg/ml for 12 h, however, did not affect IFN-γ-mediated activation of Stat1 (data not shown).

The effect of PYC on IFN-γ-induced activation of IRF-1 in HaCaT cells was also investigated. A maximum activation of IRF-1 was observed after 2 h of stimulation with 20 U/ml IFN-γ (Fig. 10A). PYC pretreatment for 12 h dose dependently suppressed IFN-γ-induced IRF-1 activation (Fig. 10B).

DISCUSSION

Keratinocyte cell adhesion

Adhesion of T cells to keratinocytes is a key feature in processes of skin inflammation [1,2]. The present study provides the first evidence that a traditional herbal medicine, PYC [13], is a potent inhibitor of IFN-γ-induced adherence of Jurkat T cells to HaCaT cells, a keratinocyte cell line. This effect of PYC on inducible cell–cell adhesion was mediated through its suppressive effect on ICAM-1 expression on HaCaT cells. De novo expression of ICAM-1 in keratinocytes is an important initiator of T cell/keratinocyte interactions during many inflammatory skin diseases [1,2]. PYC was more effective at inhibiting IFN-γ-induced cell–cell adhesion and ICAM-1 expression than any of its main constituent flavonoids that we tested.

IFN-γ-induced ICAM-1 expression

Keratinocytes have a very low constitutive expression of ICAM-1. In response to several inflammatory cytokines, ICAM-1 expression increases many-fold in these cells [24]. IFN-γ is one of the major inflammatory cytokines that induce ICAM-1 in keratinocytes, and the expression of IFN-γ is correlated with epidermal T-cell infiltration in various forms of dermatoses [24]. The spontaneously transformed human keratinocyte cell line, HaCaT [25], maintains several characteristics of cultured primary keratinocytes and is a reliable system for studying pharmacologic modulation of ICAM-1 on keratinocytes [26]. Consistent with the results of earlier studies, a marked increase in T-cell adhesion to HaCaT cells after activation of HaCaT cells with IFN-γ was observed. Upregulation of ICAM-1 expression was mainly responsible for such increased cell–cell adhesion, because treatment of HaCaT cells with ICAM-1-blocking antibody almost completely (~70%) abolished the adhesion response. The expression of ICAM-1 on HaCaT cells is upregulated dose dependently (10–500 U/ml) by IFN-γ after 4 h of treatment and reaches a plateau by 24 h [26]. In the present study, a dose-dependent increase in ICAM-1 expression consistently followed treatment with IFN-γ however, the increase in expression was not linear

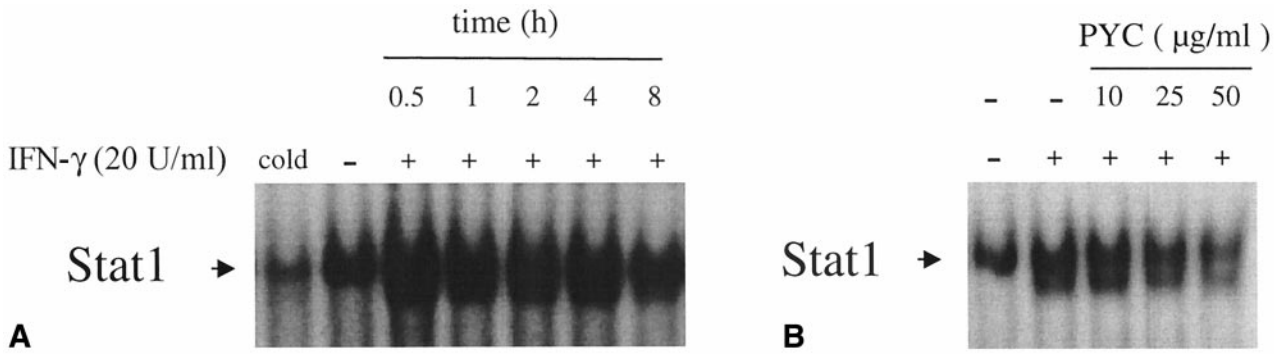

Fig. 9. Effect of PYC pretreatment on IFN-γ-mediated activation of Stat1. HaCaT cells were (A) treated with IFN-γ for different time periods (0.5–8 h) and (B) pretreated with PYC (10–50 μg/ml) and then activated with IFN-γ (20 U/ml) for 0.5 h. Nuclear translocation of Stat1 was determined by EMSA. Specificity of the DNA-binding complex was evaluated by competition with excess unlabeled Stat1 oligonucleotide.

with increasing dosages. Thus, an IFN-γ dose of 20 U/ml was selected. Moreover, this dose is more physiologically relevant compared with the higher dosages (1000 U/ml) used in other studies [8].

An intriguing observation is that TNF-α, which is a potent inducer of ICAM-1 among endothelial cells and keratinocytes [27], did not affect ICAM-1 expression on HaCaT cells. A highly variable expression of ICAM-1 in response to cytokines in keratinocytes from different donors has been observed [28]. In addition, NF-κB is a ubiquitously expressed, multisubunit transcription factor that is involved with regulation of the ICAM-1 gene expression. A strong activation in NF-κB nuclear translocation was observed in HaCaT cells after treatment with TNF-α (data not shown). Such activation, however, was not accompanied by ICAM-1 expression. Further studies are needed to address issues related to the response of TNF-α on ICAM-1 expression in HaCaT cells.

Molecular mechanisms of regulation

Flavonoids and other polyphenols have been reported to have important therapeutic potential for the treatment of various inflammatory diseases involving increased leukocyte adhesion and trafficking [19]. In addition, treatment of human endothelial cells with certain hydroxyflavones and flavanols have been reported to inhibit cytokine-induced ICAM-1 expression [19]. The antioxidant and anti-inflammatory properties of PYC, a complex mixture of bioflavonoids, has recently been reviewed [13], and among the flavonoids (i.e., taxifolin, catechin, ferulic acid) that represent its major constituents, taxifolin was the most potent for inhibiting inducible cell–cell adhesion and ICAM-1 expression. On an equal-concentration basis, PYC was the most effective for inhibiting both IFN-γ-induced cell adhesion and ICAM-1 expression compared with its individual flavonoid components. This observation suggests that various ingredients of PYC may act in co-operation to inhibit IFN-γ-induced cell–cell adhesion.

The IFN-γ-dependent induction of ICAM-1expression is regulated at the transcriptional level [29]. Stimulation of HaCaT cells by IFN-γ led to an increase in steady state ICAM-1 mRNA levels, as has been shown in other studies [30]. PYC treatment markedly suppressed the IFN-γ-mediated increase in ICAM-1 mRNA, thus

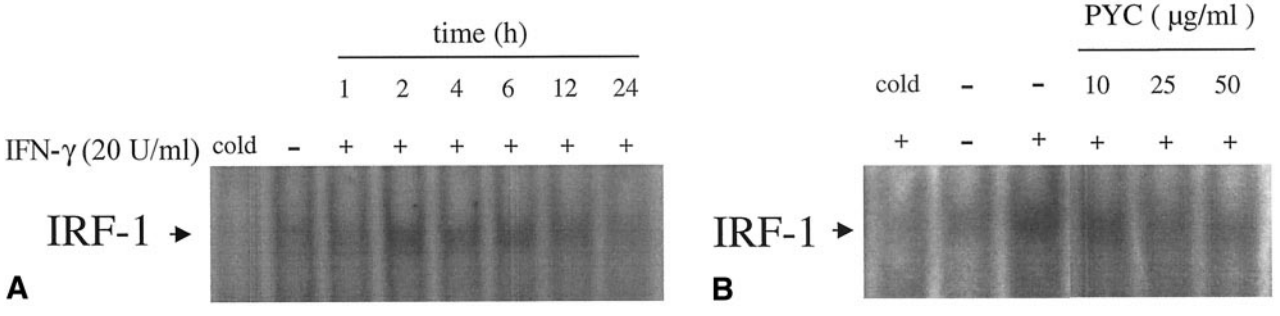

Fig. 10. Effect of PYC pretreatment on IFN-γ-mediated activation of IRF-1. HaCaT cells were (A) treated with IFN-γ for different time periods (1–24 h) and (B) pretreated with different concentrations (10–50 μg/ml) of PYC and then activated with IFN-γ (20 U/ml) for 2 h. Nuclear translocation of IRF-1 was determined by EMSA.

suggesting that PYC regulates ICAM-1 expression at the mRNA level. Such regulation could be at the transcriptional and/or the mRNA stabilization levels.

The 5 flanking region of the ICAM-1 gene contains an 11–base pair element, which is referred to as the palindromic IFN-γ response element, or pIγRE, that is located upstream of the ICAM-1 transcription initiation site between nucleotides 76 and 66. The pIγRE is composed of the sequence 5-TTTCCGGGAAA-3 [31]. The pIγRE is both necessary and sufficient for IFN-γ-dependent gene transcription [31]. The pIγRE is homologous to IFN-γ-activated sequences, which bind to tyrosine phosphorylated members of the transcription factor family known as signal transducers and activators of transcription, or (STAT) [32]. The pIγRE consists of a DNA element common to all IFN-responsive genes (GAAA) with a distinct flanking sequence (i.e., the inverted repeat GAAA) to fine tune IFN responses and activate a subset of immune response genes such as ICAM-1 or IRF-1 [32–34]. The first member of the STAT family, or Stat1, is critical for IFN-γ-dependent gene activation [31]. PYC downregulated IFN-γ-mediated activation of Stat1 in HaCaT cells dose dependently. IRF-1, which is an interferon-inducible gene, is an important component of IFN-γ signaling pathway [35]. PYC also downregulated IFN-γ-mediated IRF-1 activation in HaCaT cells. The inhibitory effect of PYC on the activation of Stat1 and IRF-1 suggests that in addition to the ICAM-1 gene, this extract also may affect expression of other IFN-responsive genes that are transcriptionally regulated by Stat1 or IRF-1.

SUMMARY

Topical application of plant extracts enriched with flavonoids as anti-inflammatory agents in patients with skin pathologies is gaining increasing attention [36–38]. Thus, a systematic characterization of the molecular mechanisms underlying the anti-inflammatory activity of flavonoids is valuable. This study provides evidence that IFN-γ-induced adhesion of T cells to keratinocytes is suppressed by PYC via inhibition of the expression of ICAM-1 on keratinocytes. The effect of PYC on inducible ICAM-1 expression may represent a mechanism that contributes to the anti-inflammatory property of this flavonoid extract. Taken together, the results presented here indicate a therapeutic potential for PYC in patients with skin pathologic conditions related to increased cell adhesion and inflammation.

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ABBREVIATIONS

DMSO—dimethylsulfoxide
DTT—dithioreitol
EDTA—ethylenediaminetetraacetic acid
EMSA—electrophoretic mobility shift assay
FITC—fluorescein isothiocyanate
GAS—gamma-activated sequence
ICAM-1—intercellular adhesion molecule-1
IFN-γ—interferon-γ
IRF-1—interferon regulatory factor-1
LFA-1—lymphocyte function-associated antigen–1
NF-κB—nuclear factor-κB
PBS—phosphate-buffered saline
pIγRE—palindromic interferon-gamma response element
PYC—pycnogenol
SSC—saline sodium citrate (0.15 M NaCl, 0.015 M sodium citrate)
SDS—sodium dodecul sulfate
Stat1—signal transducers and activators of transcription 1
TNF-α—tumor necrosis factor-alpha
UV—ultraviolet