Flavonoids differentially regulate IFNγ-induced ICAM-1 expression in human keratinocytes: molecular mechanisms of action

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Abstract The effect of plant flavonoids on intercellular adhesion molecule-1 (ICAM-1) expression in human keratinocyte was investigated. ICAM-1 is known to mediate skin inflammation. Among the flavonoids tested, taxifolin was the most potent in inhibiting interferon γ (IFNγ)-induced ICAM-1 protein as well as mRNA expression in human keratinocytes. Much smaller dosages of taxifolin were required in primary keratinocytes compared to HaCaT (immortalized cell) to achieve similar levels of inhibition in the inducible ICAM-1 expression. Regulation of inducible ICAM-1 expression by taxifolin was at transcriptional level by inhibiting the activation of signal transducers and activators of transcription (STAT)1 and protein tyrosine phosphorylation of Janus kinase (JAK)1 suggesting that the JAK–STAT pathway may be the molecular site of action of taxifolin. Finally, taxifolin pre-treatment also potently inhibited IFNγ-induced ICAM-1 expression in a reconstructed human skin equivalent suggesting therapeutic potential of taxifolin in skin pathological conditions related to increased cell adhesion and inflammation. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Cell adhesion; Taxifolin; Tyrosine kinase activation; Signal transducers and activators of transcription 1

1. Introduction

Skin inflammatory diseases are very common and often treated inadequately in spite of long continuous efforts. Only a limited number of agents such as corticosteroid, anti-histamine or immunosuppressant are available that are known to be effective in the treatment of skin inflammation. However, all of these agents have limitations, for example, anti-histamines are not as potent as other agents and may be used merely as supplemental therapy [1,2]. Corticosteroid and immunosuppressant on the other hand have potent inhibitory effects, however, only very few of these agents are suitable for long-term continuous medication [3,4]. Thus, long-term and safe control of skin inflammation is still a big challenge.

The inflammatory or immune response requires the cell–cell interaction between leukocytes and targets cells. The formation of constitutive and inducible adhesion molecule complexes is necessary for the interaction of these cells [5]. Differential expression of intercellular adhesion molecule-1 (ICAM-1), a member of the immunoglobulin gene superfamily, in the epidermis plays a critical role in the regulation of cutaneous inflammation, immunologic reactions and tissue repair. ICAM-1 mediates the firm binding of a variety of leukocytes to the target cells via its interaction with lymphocyte function-associated antigen 1 (LAF-1) or Mac-1 (CD11b/CD18) expressed on the circulating white blood cells [6,7].

Plant flavonoids are reported to have therapeutic potentials because of their anti-oxidant, anti-inflammatory, anti-cancer, anti-allergic or anti-ischemic properties [8–15]. Most of the biological activity of flavonoids is attributed to their redox modulatory and protein kinase inhibitory properties. We have recently shown that the flavonoid quercetin potently inhibits inducible ICAM-1 expression in endothelial cells by inhibiting the Jun-N-terminal kinase pathway [16]. Furthermore, pine bark extract, a complex flavonoid, also inhibited ICAM-1 expression in HaCaT cells by inhibiting signal transducers and activators of transcription (STAT) 1 activation [17]. In vivo, skin penetration studies of flavonoids provide evidence that the flavonoids after topical application can be absorbed from the skin surface and penetrate into deeper skin layers [18]. Katiyar et al. observed that topical application of (−)-epigallocatechin-3-gallate (3 mg/2.5 cm^2), the major polyphenolic constituent in green tea, before UVB (4 MED) exposure to human skin significantly blocked UVB-induced infiltration of leukocytes [19]. The present study was aimed to investigate the effect of representative compounds of various flavonoid classes on inducible ICAM-1 expression. Furthermore, the molecular site of regulation of ICAM-1 by flavonoids was also investigated.

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Abbreviations: ICAM-1, intercellular adhesion molecule-1; IFNγ, interferon γ; JAK1, Janus kinase 1; STAT1, signal transducers and activators of transcription 1

2. Materials and methods

2.1. Cells and cell culture

The immortalized human keratinocyte line HaCaT was grown in Dulbecco's modified Eagle's medium (Life Technologies, Gaithers-

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burg, MD, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. Primary human epidermal keratinocytes (HEK) were obtained from a 25-year-old male (Cascade Biologics, Portland, OR, USA). HEK were grown in Medium 154 supplemented with human keratinocyte growth supplement and PSA (penicillin, streptomycin and amphotericin B) solution (Cascade Biologics). Human Jurkat T-cells clone E6-1 (American Type Culture Collection, Bethesda, MD, USA) were grown in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin, 110 mg/l sodium pyruvate and 2 mM L-glutamine. Cells were pre-treated with various classes of flavonoids (Fig. 1) and then activated with interferon γ (IFNγ). Taxifolin and other flavonoid stock solutions for cell treatment were prepared fresh in dimethyl sulfoxide (DMSO) at concentrations such that the final concentration of the solvent in cell suspension never exceeded 0.1%. A three-dimensional (3D) model of HEK (EpiDerm; Kurabo, Osaka, Japan) was used for immunohistochemical experiments. This model consists of normal human-derived epidermal keratinocytes, grown on permeable cell culture inserts that have the potential to form multilayered and highly differentiated keratinocytes [20]. The EpiDerm assay kit contains 24 units of 8-mm diameter tissue samples. The epidermal equivalents were removed from the 24-well plates containing agarose and equilibrated to 37°C, 5% CO_2 for 1 h with the assay medium by the vendors. The epidermal equivalents were then transferred under sterile conditions to a petri dish containing phosphate-buffered saline (PBS) for subsequent treatments.

2.2. Expression of ICAM-1

The HaCaT cells or HEK were washed in Dulbecco's PBS, pH 7.4 (D-PBS) and incubated with fluorescein isothiocyanate (FITC) coupled to ICAM-1 monoclonal antibody (Immunotech, Marseille, France) for 30 min at 4°C and washed once in D-PBS and finally resuspended in fresh D-PBS. Expression of ICAM-1 was immediately assayed with a flow cytometer. Appropriate isotypic controls were used for background fluorescence in ICAM-1 assay. Flow cytometric analyses were performed using the fluorescence and light scattering properties (forward scatter, FS; side scatter, SS) as described before [21].

2.3. Cell adhesion assay

Cell–cell adhesion was performed as previously described [21,22]. Briefly, monolayers of HaCaT cells or HEK 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). Calcein-AM (Molecular Probes, Eugene, OR, USA)-labeled Jurkat T-cells (3×10^5 cells/well) were co-cultured with HaCaT cells or HEK monolayers for 1 h in a culture incubator with humidified air containing 5% CO_2 at 37°C. The fluorescence intensity of each well was measured using a fluorescence plate reader. The excitation and emission 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 or HEK was evaluated by treating HaCaT cells or HEK with anti-human ICAM-1 (10 μg/ml) antibody (Beckman Coulter, Fullerton, CA, USA) for 30 min prior to co-culture with Jurkat T-cells.

2.4. Real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from HaCaT cells or HEK using guanidium isothiocyanate following the method of Chomczynski and Sacchi [23]. Amplification reactions were performed with the TaqMan method (Perkin-Elmer Applied Biosystems, CA, USA). Primers and the TaqMan probe for ICAM-1 were designed using the primer design software Primer Express TM (Perkin-Elmer Applied Biosystems). The sequences of the primers and TaqMan probes used in this study are ICAM-1 forward primer; 5′-GCAATGTGCAAGAAGATAGCCA-3′, reverse primer; 5′-GTGAGGAAGGTTTTAGCTGTTGACTG-3′, probe; 5′-CCAATGTGCTATTCAAACTGCCCTGATGG-3′, GAPDH forward primer; 5′-GAAGGTGAAGGTCGGAGT-3′, reverse primer; 5′-GAAGATGGTGATGGGATTTC-3, probe; 5′-CAAGCTTCCCGTTCTCAGCC-3′. Amplification of glyceraldehyde-3-phosphate dehydrogenate (GAPDH) (TaqMan GAPDH control reagent kit) was used as an endogenous control for quantification.

2.5. Electrophoretic mobility shift assay (EMSA)

The nuclear extracts were prepared and EMSAs were performed essentially as described previously [21]. Binding reaction mixtures (20 μl) containing: 5 μg protein of nuclear extract, 2 μg poly(dI-dC) (Pharmacia, Sweden), ^{32}P-labeled STAT1 probe (Santa Cruz, CA, USA), 50 mM NaCl, 2 mM $MgCl_2$, 0.2 mM Na_2EDTA, 1 mM dithiothreitol (DTT), 10% (v/v) glycerol and 4 mM Tris–HCl (pH 7.9) were incubated for 30 min at room temperature. Proteins were separated by 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.

2.6. Immunoprecipitation and immunoblot analysis

HaCaT cells were washed with ice-cold D-PBS containing 200 μM sodium orthovanadate (Na_3VO_4), frozen immediately in liquid nitrogen, and then lysed in lysis buffer (25 mM Tris–HCl, pH 7.6, 200 mM boric acid, 150 mM NaCl, 50 mM NaF, 5 mM Na_2EDTA, 1% Triton X-100, 10 mM sodium pyrophosphate, 2 mM EGTA, 20 mM *p*-nitrophenyl phosphate, 1% bovine serum albumin, 20 μM Na_3VO_4, 2 mM DTT) containing protease inhibitors (2 μg/ml each of aprotinin, leupeptin, pepstatin, antipain and 100 μg/ml of phenylmethylsulfonyl fluoride). Samples (20 μg of protein each) were separated on 10% sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) and blotted onto Hybond-ECL nitrocellulose membranes (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The membranes were blocked with 5% skim milk for 2 h at room temperature, and then probed with a horseradish peroxidase (HRP)-conjugated anti-phosphotyrosine antibody, PY20 (Santa Cruz, CA, USA) overnight at 4°C. Bound antibody was detected using the enhanced chemiluminescence Western blotting detection reagent (Amersham Pharmacia Biotech). Supernatants for immunoprecipitation were incubated at 4°C with the anti-Janus kinase 1 (anti-JAK1) antibody (Santa Cruz, CA, USA) bound to G-Sepharose beads (2 μg of antibody per 20 μl of beads; Amersham Pharmacia Biotech) for 4 h, after which the beads were washed twice with 1 ml of WG buffer (50 mM HEPES-NaOH, pH 7.6, 150 mM NaCl, 0.1% Triton X-100) and resuspended in SDS sample buffer.

2.7. Immunohistochemistry

Following topical application of taxifolin (20 μM) or DMSO, EpiDerm were activated with IFNγ 1000 U/ml for 12 h, and then the tissues were immediately embedded in paraffin. Slides were stained with a streptavidin–biotin–peroxidase complex procedure using the Dako CSA System, HRP (Dako, CA, USA) according to the manufacturer's protocol. In brief, the sections were incubated at room temperature for 30 min with an anti-human ICAM-1, CD54 (Dako). After three washes with PBS, the samples were incubated with biotinylated anti-mouse IgG and the avidin–biotin complex at room temperature for 15 min. Staining was completed by a 5-min incubation with 3,3′-diaminobenzidine tetrahydrochloride.

2.8. Statistical analysis

Data are presented as mean ± S.D. of at least three separate experiments. Differences between the means of groups were determined by Student *t*-test and analysis of variance. The minimum level of significance was set at $P<0.05$.

3. Results

3.1. IFNγ-induced expression of ICAM-1 in keratinocytes

Treatment of cells with IFNγ for 24 h resulted in increased expression of ICAM-1 in HaCaT cells or primary HEK (Fig. 1). We have previously reported that IFNγ dose-dependently (20–300 U/ml) induces ICAM-1 expression in HaCaT cells [17]. Basal ICAM-1 expression levels were very low in unstimulated cells. In subsequent experiments a 20 U/ml dose of IFNγ was used. Treatment (24 or 48 h) of HaCaT cells or HEK with tumor necrosis factor-α, which is a potent inducer of ICAM-1 in endothelial cells, did not affect the expression of ICAM-1 (Fig. 1A). The effect of various flavonoids on IFNγ-induced ICAM-1 expression in HaCaT cells was investigated. Among all the flavonoids tested, taxifolin (100 μM, 12 h) was observed to be the most potent in inhibiting induc-

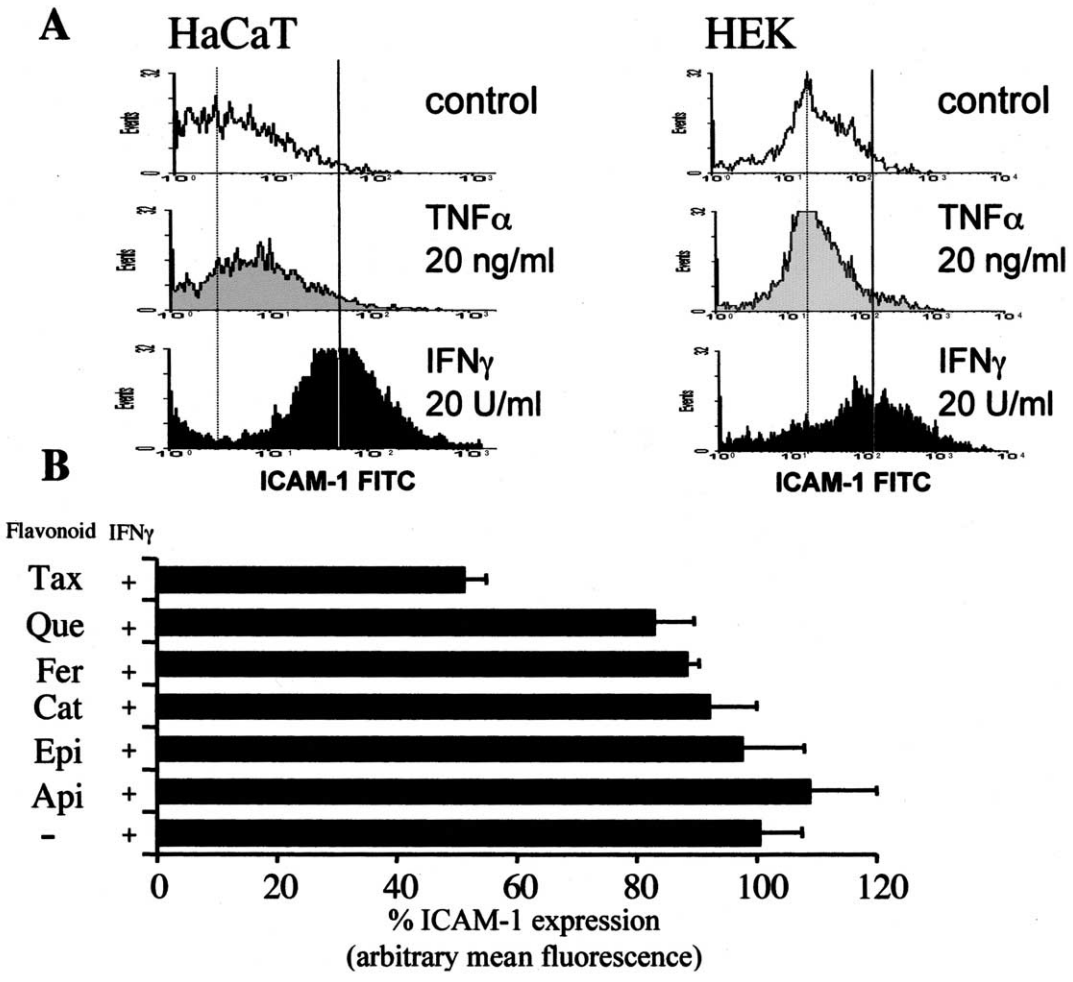

Fig. 1. Flavonoids down-regulate IFNγ-induced ICAM-1 expression in HaCaT. A: Flow cytometric analysis of the HaCaT cells and HEK activated with IFNγ (20 U/ml, 24 h) or TNF-α (20 ng/ml, 48 h). Broken and solid lines represent median value of log ICAM-1 FITC signal of control (no treatment) cells and IFNγ-treated cells, respectively. Data are presented as histograms of cell number versus log ICAM-1 FITC fluorescence intensity. Histograms are representative of three identical experiments. B: HaCaT cells were pre-treated with equal concentrations (100 μM) of Api (apigenin, anthocyanidins), Qrct (quercetin, flavonol), Cat (catechin, flavanol), Fer A (ferulic acid, phenyl propanoids), Epicat (epicatechin, flavanol), or Tax (taxifolin, flavanone) for 12 h, followed by activation with IFNγ (20 U/ml) for 24 h. ICAM-1 expression was measured using a flow cytometer. Data are presented as percent compared to the IFNγ-activated cells. Data are mean ± S.D. of at least three experiments.

ible ICAM-1 expression in HaCaT cells (Fig. 1B). To characterize the kinetics of taxifolin-mediated inhibition of IFNγ-induced expression of ICAM-1, HaCaT cells were pre-treated with 100 μM taxifolin for different time periods followed by activation with IFNγ for 24 h. Maximal inhibition (~60% compared to non-treated activated cells) of inducible ICAM-1 expression was observed in cells that were pre-treated with taxifolin for 12 h (data not shown). Such down-regulation of IFNγ-induced ICAM-1 expression by taxifolin was dose-dependent in HaCaT cells (5–100 μM) as well as HEK (1–20 μM) (Fig. 2A). The effect was already significant ($P < 0.05$) in HaCaT cells or HEK with the 25 or 5 μM dose of taxifolin pre-treatment, respectively. A lower concentration of taxifolin was required in HEK cells to achieve a similar degree of inhibition as that achieved in HaCaT cells (Fig. 2A).

The non-permeant DNA intercalating dye propidium iodide (Molecular Probes) was used to determine the viability of HaCaT cells or HEK following treatment of the cells with various doses of taxifolin [24]. The concentrations (up to 100 μM for HaCaT; 20 μM for HEK) of taxifolin were observed to be not cytotoxic to the cells (data not shown).

3.2. *Taxifolin inhibits IFNγ-induced adhesion of Jurkat cells to keratinocytes*

The co-culture assay was performed to verify whether the inhibitory effect of taxifolin on the expression of ICAM-1 was also effective in down-regulating actual cell to cell adhesion. Pre-treatment of taxifolin (50 or 100 μM) for 12 h significantly inhibited the IFNγ-induced adherence of Jurkat T-cells to HaCaT cells (Fig. 2B). Compared to the non-treated activated group, a 60% inhibition in cell adhesion was observed in cells that were pre-treated with taxifolin (100 μM). Consistent with the inhibitory effect of taxifolin on inducible ICAM-1 expression in HEK, taxifolin pre-treatment dose-dependently (1–20 μM) inhibited IFNγ-induced adherence of Jurkat cells to HEK (Fig. 2B). Treatment of the cells prior to the adhesion assay with ICAM-1 antibody inhibited 60–70% of the adherence of Jurkat T-cells to HaCaT cells or HEK suggesting a

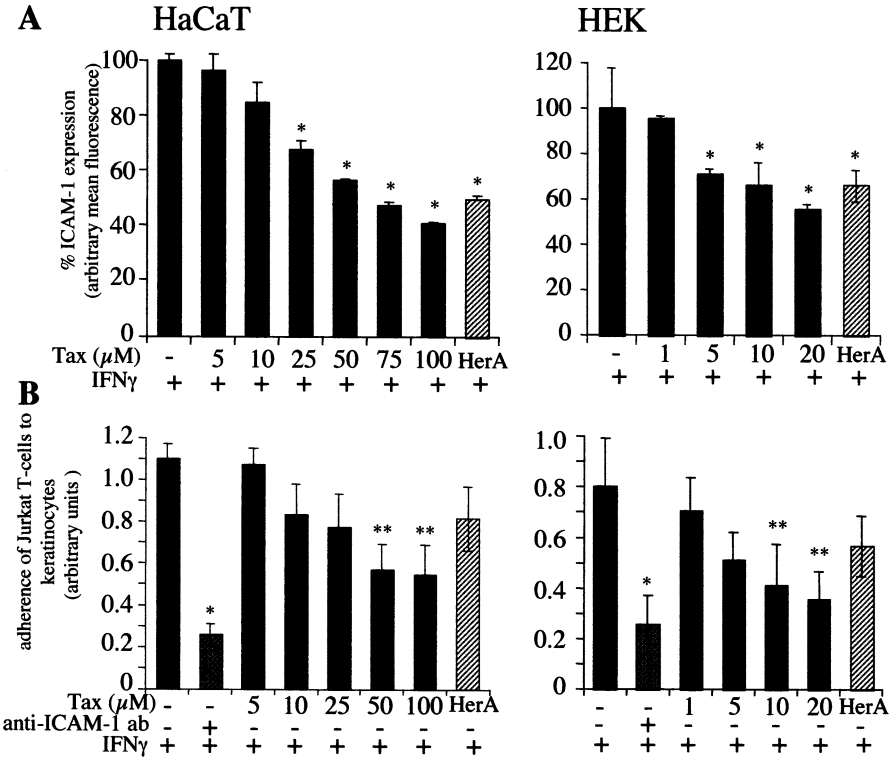

Fig. 2. Effect of taxifolin pre-treatment on IFNγ-induced ICAM-1 expression and cell adhesion. A: HaCaT cells or HEK were pre-treated with taxifolin for 12 h or herbimycin A (1 μM) for 15 min followed by activation with IFNγ (20 U/ml) for 24 h. Data are mean ± S.D. of at least three experiments. B: HaCaT cells or HEK were pre-treated with taxifolin for 12 h or herbimycin A (1 μM) for 15 min, and then activated with 20 U/ml IFNγ for 24 h. The cells were washed three times with D-PBS and then co-cultured with calcein-AM-labeled Jurkat T-cells for 1 h. The relative contribution of ICAM-1 in inducible cell–cell adhesion was determined by incubating cells with ICAM-1 blocking antibody (10 μg/ml) for 30 min prior to the assay. Data are mean ± S.D. of at least three experiments. $^{*}P < 0.01$ or $^{**}P < 0.05$ when compared with taxifolin non-treated IFNγ-activated group.

major role of ICAM-1 in IFNγ-induced adhesion of Jurkat cells to keratinocytes (Fig. 2B).

3.3. Transcriptional regulation of IFNγ-induced ICAM-1 expression

ICAM-1 mRNA was markedly increased in HaCaT cells or HEK treated with IFNγ (20 U/ml) for 4 h. Taxifolin pre-treatment inhibited such an increase in ICAM-1 mRNA (Fig. 3A). To investigate whether the inhibitory effects of taxifolin on inducible ICAM-1 mRNA expression is due to faster degradation of the mRNA by taxifolin, the stability of ICAM-1 mRNA was studied. The cells were stimulated with IFNγ alone for 4 h, and then actinomycin D (5μg/ml) was added to prevent further mRNA synthesis in the absence or presence of taxifolin (100 μM). No significant difference in the rates of degradation of ICAM-1 mRNA in the presence or absence of taxifolin was observed, indicating that the inhibitory effect of taxifolin on inducible ICAM-1 mRNA expression is not due to the faster degradation of ICAM-1 mRNA by taxifolin (Fig. 3B).

The binding motif of the γ-activated sequence has been identified in ICAM-1 promoter. Following activation, translocation of the STAT1 protein to nucleus and binding to the γ-activated sequence motif plays a major role in the transcriptional activation of IFNγ-induced ICAM-1 gene expression [25]. The effect of taxifolin on activation and nuclear translocation of STAT1 was investigated by EMSA. IFNγ treatment to HaCaT cells resulted in activation and nuclear translocation of STAT1. A maximal increase in the nuclear translocation of STAT1 was observed following 30 min of IFNγ (20 U/ml) treatment [17]. Taxifolin pre-treatment for 12 h dose-dependently (10–100 μM) decreased IFNγ-mediated activation of STAT1 in HaCaT cells (Fig. 4A). In HEK, the dose (20 μM, 12 h) of taxifolin that is required to inhibit inducible ICAM-1 protein and mRNA expression also effectively inhibited the IFNγ-mediated activation of STAT1 (Fig. 4A).

3.4. Tyrosine phosphorylation regulates IFNγ-induced ICAM-1 expression

Activation of IFNγ-responsive genes requires tyrosine phosphorylation of the transcriptional factor STAT1. Tyrosine kinase inhibitor, herbimycin A was used to investigate the role of tyrosine phosphorylation in the regulation of IFNγ-induced ICAM-1 expression in HaCaT cells and HEK. Herbimycin A (1 μM) pre-treatment for 15 min to HaCaT cells or HEK markedly inhibited IFNγ-induced ICAM-1 expression as well as the inducible adherence of Jurkat cells to keratinocytes (Fig. 2). Herbimycin A pre-treatment also potently inhibited the IFNγ-induced ICAM-1 mRNA expression (Fig. 3A) and STAT1 activation (data not shown). The role of reactive oxygen species in the pathway was also explored using potent anti-oxidants such as α-lipoate or *N*-acetylcysteine, however, the suppressive effect achieved by these anti-oxidants on the

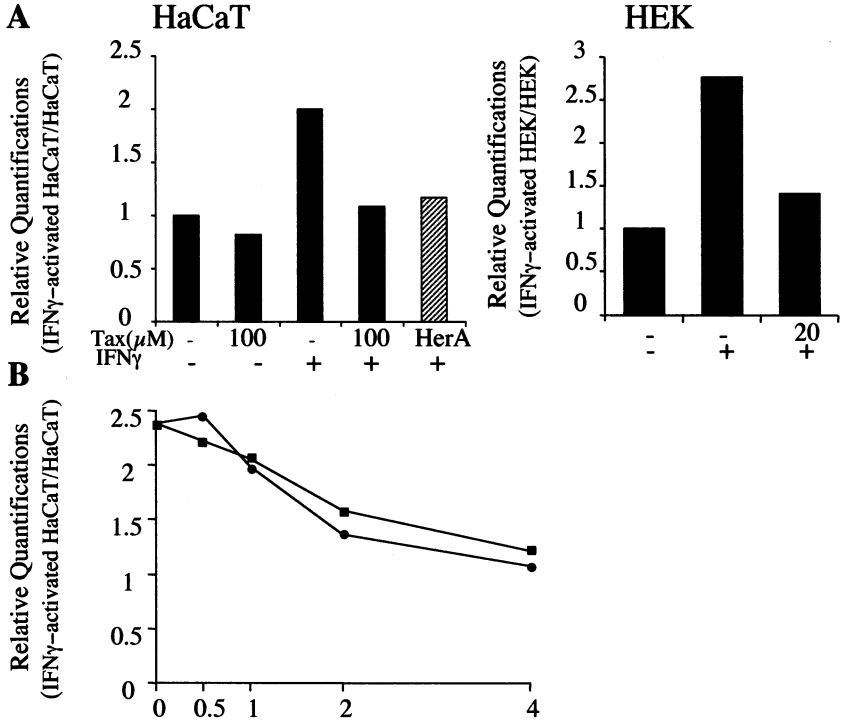

Fig. 3. Effect of taxifolin on IFNγ-activated ICAM-1 mRNA. A: Cells were pre-treated with taxifolin for 12 h and then activated with IFNγ (20 U/ml) for 4 h. HaCaT cells were also pre-treated with herbimycin A (1 μM) for 15 min and then activated with IFNγ. B: HaCaT cells were treated with IFNγ (20 U/ml) for 4 h, and then treated further with actinomycin D (Act D; 5 μg/ml), either alone (■) or with taxifolin (●, 100 μM) for time period indicated (hour). Relative mRNA levels of ICAM-1 were determined by quantitative RT-PCR of HaCaT cells or HEK with or without activation of IFNγ. Expression of ICAM-1 mRNA is in arbitrary units, and data are normalized to non-activated cells. GAPDH was selected as an endogenous RNA control to normalize for differences in the amount of total RNA. Data are representative of three independent experiments.

IFNγ-induced ICAM-1 expression was much smaller compared to that by herbimycin A or taxifolin (not shown). Herbimycin A data suggested that protein tyrosine phosphorylation was involved in the transcriptional regulation of IFNγ-induced ICAM-1 expression in HaCaT cells and HEK. Thus, to confirm whether the mode of action of taxifolin in inhibiting inducible ICAM-1 is via affecting the inducible tyrosine phosphorylation, the cells were pre-treated with taxifolin and then activated with IFNγ. Overall protein tyrosine phosphorylation was investigated by Western blot using phosphotyrosine specific antibody. IFNγ (20 U/ml) activation induced protein tyrosine phosphorylation in HaCaT cells. Specifically, one major band around 130 kDa was heavily phosphorylated following IFNγ treatment. Maximal activation in this band was observed following 15 min exposure to IFNγ. Taxifolin (100 μM) treatment of the cells for 12 h resulted in a marked inhibition of IFNγ-induced phosphorylation of the 130 kDa band (data not shown). Since the molecular weight of JAK1, JAK2 or Tyk2, which mediates IFNγ signaling, is around 130 kDa, phosphorylation of the tyrosine residue of these kinases was analyzed by immunoprecipitation. Immunoprecipitation with anti-JAK1, anti-JAK2 or anti-Tyk2 antibody and blotting with anti-phosphotyrosine antibody (PY20) confirmed that the 130 kDa band is JAK1 protein (Fig. 4B), because we could not detect the phosphorylation of JAK2 or Tyk2 by IFNγ in HaCaT cells. Thus, JAK1 appeared to be a potential candidate for taxifolin-mediated down-regulation of IFNγ-induced phosphorylation.

3.5. Taxifolin suppressed IFNγ-induced ICAM-1 expression in a reconstructed human skin model

To verify physiological relevance of taxifolin treatment in inflammatory skin disorders, the effect of taxifolin on IFNγ-induced ICAM-1 expression was evaluated in a reconstructed human skin model (EpiDerm). Because of a plastic cup that surrounds the 3D human skin model it is possible to regulate the actual volume of the medium as well as the exact concentration of reagents applied to the model. To obtain sufficient ICAM-1 expression levels in EpiDerm that are detectable by immunohistochemical analysis, a relatively high dose (1000 U/ml) of IFNγ was used. Regardless of the high dose of IFNγ used for ICAM-1 expression, topical application of taxifolin as low as 20 μM for 12 h markedly suppressed IFNγ-induced ICAM-1 expression in EpiDerm (Fig. 5).

4. Discussion

Adhesion of T-cells to keratinocytes is a key feature in skin inflammation processes [26,27]. The present study provides the first evidence that among various flavonoids tested, taxifolin is a potent inhibitor of IFNγ-induced adherence of Jurkat T-cells to keratinocytes. Such effect of taxifolin on inducible cell–cell adhesion was mediated via its inhibitory effect on ICAM-1 expression. The maximum effect of taxifolin on inducible ICAM-1 was observed following 12 h of pre-treatment suggesting that native taxifolin is not active in inhibiting inducible ICAM-1 expression. A 12 h period required to achieve

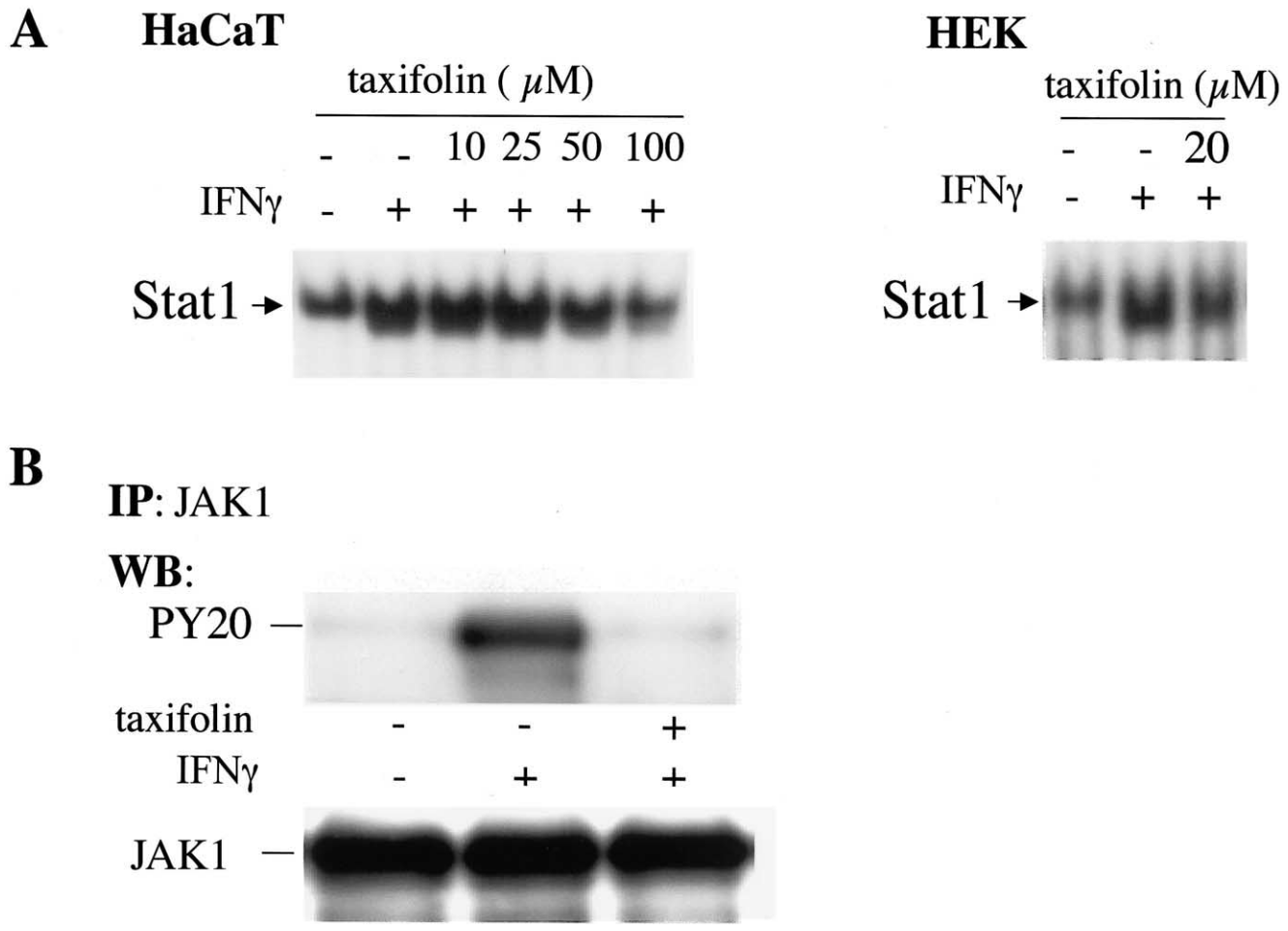

Fig. 4. Effect of taxifolin pre-treatment on IFNγ-mediated activation of STAT1 and JAK1. A: HaCaT cells were pre-treated with taxifolin (10–100 μM) for 12 h and then activated with IFNγ (20 U/ml) for 0.5 h; and HEK was also pre-treated with taxifolin (20 μM) and then activated with IFNγ (20 U/ml) for 0.5 h. Nuclear translocation of STAT1 was determined by EMSA. The specificity of the DNA binding complex was evaluated by competition with excess unlabeled (cold) STAT1 oligonucleotide. B: HaCaT cells were pre-treated with taxifolin (100 μM) for 12 h and then activated with 20 U/ml IFNγ for 15 min. Cell lysates were then extracted from IFNγ-stimulated and unstimulated HaCaT cells and subjected to immunoprecipitation (IP) with the anti-JAK1 antibody. The immunoprecipitates were run on a SDS–PAGE and subjected to immunoblot analysis with PY20. The same membrane was then reprobed with the anti-JAK1 antibody. Data are representative of three independent experiments.

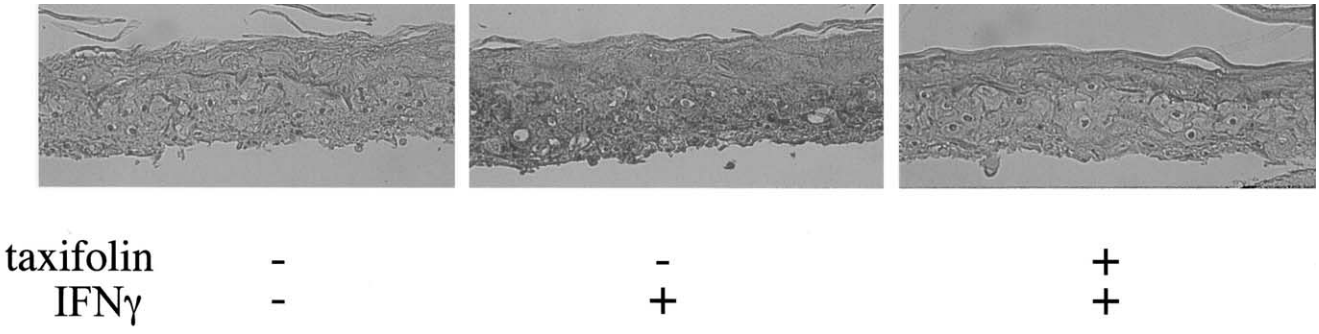

Fig. 5. Topical application of taxifolin on IFNγ-induced ICAM-1 expression in a 3D model of human skin epidermis. EpiDerm were treated with DMSO (0.5% v/v) or taxifolin (20 μM) for 12 h, and then incubated with 1000 U/ml IFNγ for 12 h. ICAM-1 expression is seen in the cytoplasm of most keratinocytes of IFNγ treated EpiDerm (middle panel), whereas little ICAM-1 expression is seen in non-treated EpiDerm (left panel). Topical application of taxifolin suppressed IFNγ-induced ICAM-1 expression (right panel). Left panel: DMSO only; middle panel: DMSO+IFNγ; right panel: taxifolin+IFNγ. Data are representative of two independent experiments.

the maximal inhibitory effect on ICAM-1 may be due to the rate of uptake of taxifolin in cells or cellular activation/transformation of taxifolin being required to achieve the observed effect. The study also provides the first evidence that topical application of a low concentration (20 μM) of a naturally occurring flavonoid taxifolin markedly inhibits IFNγ-induced ICAM-1 expression in a reconstructed human skin model. The observation that much lower dosages of taxifolin are required in HEK (primary cells) to achieve the comparable effects on inducible ICAM-1 expression observed in HaCaT suggests that data obtained using immortalized cells should be interpreted with caution in terms of the effective concentration.

IFNγ-dependent induction of ICAM-1 expression is regulated at the transcriptional level [28]. Marked suppression of the IFNγ-mediated increase in ICAM-1 mRNA by taxifolin without affecting the stability of ICAM-1 mRNA level suggests that regulation of inducible ICAM-1 expression in keratinocytes is at a transcriptional level. The 5′-prime flanking region of ICAM-1 gene contains an 11-basepair element, which is referred to as the palindromic IFNγ response element (RE), or pIγRE, located upstream of the ICAM-1 transcription initiation site between nucleotides −76 and 66. The pIγRE is composed of the sequence 5′-TTTCCGGGAAA-3′ [29]. It has been demonstrated that pIγRE is both necessary and sufficient for IFNγ-dependent gene transcription [29]. pIγRE is homologous to IFNγ-activated sequences, which bind to tyrosine phosphorylated members of the transcription factor family known as STAT [30]. pIγRE consists of a DNA element common to all IFN-responsive genes (GAAA) with a distinct flanking sequence (the inverted repeat GAAA) in order to fine-tune IFN responses and activate a subset of immune response genes such as ICAM-1 or IRF-1 [30–32]. The first member of the STAT family (designated as STAT1) is critical for IFNγ-dependent gene activation [29]. Taxifolin dose-dependently inhibited nuclear translocation of STAT1 in HaCaT cells as well as HEK.

IFNγ, a homodimeric molecule, bivalently binds to its receptor and activates the JAK/STAT pathway by clustering its receptor. The Janus family of tyrosine kinases is an integral component of IFNγ-activated signaling cascades, which regulate tyrosine phosphorylation of the STAT proteins such that they can translocate to the nucleus and bind DNA [33]. IFNγ stimulates tyrosine phosphorylation within the cytoplasmic domain of its receptor this event occurs concomitantly with tyrosine phosphorylation of both JAK1 and JAK2 [25]. A distinct band of 130 kDa was heavily phosphorylated following IFNγ activation. Probing the membrane with anti-JAK1 antibody demonstrated that the 130 kDa band is JAK1 protein. Taxifolin significantly inhibited tyrosine phosphorylation of this 130 kDa band suggesting that one of the potential sites of action of taxifolin in inhibiting inducible ICAM-1 is by down-regulating phosphorylation of JAK1. As the similar effects were observed by herbimycin A treatment, further mechanism of action of taxifolin which is distinguishable from that of herbimycin A will be of interest for future study.

Regulation of ICAM-1 gene expression has been related to oxidative stress through specific reduction–oxidation (redox) sensitive transcriptional regulation [34]. The inhibitory effect of taxifolin on inducible ICAM-1 expression may be due to its anti-oxidant activity. The analysis of structure–anti-oxidant activity relationships using Trolox equivalent anti-oxidant capacity has demonstrated that taxifolin has less anti-oxidant potential than that of the other flavonoids such as quercetin [35] that did not significantly change inducible ICAM-1 expression in keratinocytes. These data suggest that it is less likely that the anti-oxidant activity of taxifolin is mainly responsible for its inhibitory effect on the inducible ICAM-1 expression though the contribution of redox state may not be ruled out completely.

De novo expression of ICAM-1 in keratinocytes is an important initiator of T-cell/keratinocyte interactions in many inflammatory skin diseases [26,27]. The marked inhibition of IFNγ-induced ICAM-1 expression by taxifolin was observed in a 3D human skin model. In conclusion this study provides therapeutic potential of this flavonoid in skin pathological conditions related to increased cell adhesion and inflammation.

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