Immunomodulatory effects of phytocompounds characterized by in vivo transgenic human GM-CSF promoter activity in skin tissues

Pei-Fen Su · Vanisree Staniforth · Chin-Jin Li · Chien-Yu Wang · Ming-Tsang Chiao · Sheng-Yang Wang · Lie-Fen Shyur · Ning-Sun Yang

Received: 28 March 2008 / Accepted: 22 June 2008 / Published online: 13 July 2008 © National Science Council Taipei 2008

Abstract To investigate the immunomodulatory activities of phytocompounds for potential therapeutics, we devised an in vivo, transgenic, human cytokine gene promoter assay using defined epidermal skin cells as test tissue. Test compounds were topically applied to mouse skin before or after gene gun transfection, using a cytokine gene promoter-driven luciferase reporter. Croton oil, an inflammation inducer, induced transgenic GM-CSF and TNF-α promoter activities in skin epidermis 6-fold and 3.4-fold, respectively; however, it produced a less than 1.5-fold and 1.7-fold change in IL-1β and IL-18 promoter activity, respectively. The phytocompound shikonin drastically inhibited inducible GM-CSF promoter activity. However, a fraction of Dioscorea batatas extract significantly increased the GM-CSF promoter activity in normal and inflamed skin. Shikonin suppressed the transcriptional activity of GM-CSF promoter by inhibiting the binding of TFIID protein complex (TBP) to TATA box. Our results demonstrate that this in vivo transgenic promoter activity assay system is cytokine gene-specific, and highly responsive to pro-inflammatory or anti-inflammatory stimuli. Currently it is difficult to profile the expression and cross-talk of various types of cytokines in vivo. This investigation has established a bona fide in vivo, in situ, immune tissue system for research into cytokine response to inflammation.

Keywords Dioscorea batatas · Immune-modulating activities · In vivo assay · Human granulocyte macrophage-colony stimulating factor · Phytocompounds · Shikonin

Introduction

Cytokines, the key regulators of the immune system, have been studied intensively as a class of powerful immunomodulators for clinical applications. However, the adverse side effects, high cost, and labile features of cytokine proteins often prohibit their routine use. Efforts are therefore underway to develop alternative immunomodulators as therapies. One source of alternative immunomodulators may be traditional herbs or their derived phytocompounds reputed to confer medicinal efficacy [1]. The historically successful development of medicinal compounds from plants, such as aspirin and taxol, has led to research into the potential of a large range of herbs and their derived phytocompounds as a source of immunomodulators [2, 3].

In drug discovery research, in vitro cell-based screening systems are well established as methods for evaluation of candidate lead compounds. For example, in vitro assays of NF-κB [4, 5] and COX-2 [6], two examples of drug targets, are employed to develop therapeutic strategies to counter inflammation. However, it is also known that the regulation of immune-modifiers and their gene expression is highly dependent upon three dimensional microenvironments. Therefore, an in vivo assay that can accurately evaluate the effects of immune modulators/drugs on the expression
level of key cytokines in targeted tissue is highly desirable for applications in medical biotechnology. Keratinocytes are known as the major source of cytokines in skin tissues [7, 8], and these cells have also been shown to be the skin cell type most susceptible to gene transfection with a gene gun [9]. The purpose of this study was to establish the use of promoter activities from a panel of cytokine genes as the basis for a reporter system that can characterize the transcriptional control of cytokine gene(s) responsive to topically applied pro- or/and anti-inflammatory phytocompounds.

GM-CSF (granulocyte macrophage-colony stimulating factor) is a cytokine known for its multi-functional effects on various immune cell systems. Recombinant human GM-CSF is used therapeutically for enhancing hematopoietic recovery [10] and anti-infection activities [11]. Recently, GM-CSF has also been actively evaluated as an immune adjuvant to enhance maturation and function of dendritic cells and to augment macrophage activities [12]. GM-CSF is also applied as a vaccine adjuvant in various immunotherapy approaches, including cancer treatment [13–16]. However, dysregulated GM-CSF has been shown to be associated with chronic inflammatory diseases such as cutaneous dermatitis [17, 18] and skin cancer [19]; and has thus been suggested as a therapeutic target for multiple sclerosis [20]. Therefore, human GM-CSF was chosen as the key cytokine for this study.

A number of candidate immunomodulatory agents, including medicinal herb extracts of traditional Chinese medicine and Western remedies, purified phytocompounds, and anti-inflammatory prescription drugs were quantitatively analyzed for pro- or anti-inflammatory activities in this study. Since different clusters of specific cytokine genes are related to different types of skin inflammation [21], to evaluate various cytokine gene responses to this system. Correlations between inflammatory responses, specific cytokine gene promoter activities, and inhibition or enhancement of these activities by specific phytocompounds or herbal extracts were elucidated. Information from this study provides a quantifiable and quick in vivo assay system for validating and characterizing potential immunomodulators (e.g., phytocompounds) as candidate therapeutics.

### Materials and methods

#### Plasmids

Human genomic DNA was isolated from lymphocytes using a Genomic DNA purification kit (Promega, Madison, WI) and was then used as a template for amplifying the promoter region of target cytokine genes by an expanded long template PCR system (Roche, Mannheim, Germany). The sequences of target promoter regions of cytokine genes were obtained from either published results or the NCBI database. For GM-CSF, a proximal promoter fragment of 620-bp (−620 to −1) and another fragment of 3.3-kb (−3286 to −1) including the distal enhancer region were PCR-amplified and individually cloned into a pGL-3 basic (Promega), which is a luciferase reporter vector. The PCR-amplified promoter sequences of IL-1β (881 bp, −853 to +28), IL-18 (909 bp, −738 to +171), and TNF-α (1097 bp, −1049 to +48) were also individually cloned into pGL3-basic vector as depicted in Table 1. The proximal promoter motif of GM-CSF was also cloned into the pβ-Gal basic vector (Clontech, Palo Alto, CA) to generate a pGM620-β-gal plasmid with a β-galactosidase reporter gene driven by GM-CSF promoter.

#### Mice

Female BALB/c Byj mice aged 8–12 weeks, were purchased from the National Laboratory Animal Breeding and Research Center, Taipei, Taiwan and maintained under standard pathogen-free, animal room conditions.

<p>| Table 1 Primer sequences and cloning sites of cytokine promoters amplified by PCR |
|----------------------------------|------------------------------------------|------------------------------|------------------|</p>
<table>
<thead>
<tr>
<th>Promoter of gene</th>
<th>Primer sequences (5’ → 3’)</th>
<th>Cloning site</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>CGGGGTACCCTGGACGGCAGCAGCTTTGCTCTGTGC<em>C</em>a</td>
<td>KpnI/BglII</td>
<td>[35]</td>
</tr>
<tr>
<td></td>
<td>GAGATCTTGCTTGGCTCCTGAAGAAGGTGTTG*b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-18</td>
<td>AGTCCTAAGCCTTTGCTATCTATCCAGGAA**a</td>
<td>SacII/NheI</td>
<td>[37]</td>
</tr>
<tr>
<td></td>
<td>GTACGTACGTCTCTCTCTCTCTCTAGGAACTG*b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>CGGACGCGTGAAAGCAAGAGAGAGAGTGCTGAAGAAGA**a</td>
<td>MluI/BglII</td>
<td>L11698</td>
</tr>
<tr>
<td></td>
<td>CGGAGATCTCTCCTAGTCTCTCTCCTAGTCGTC*b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GM-CSF (620 bp)</td>
<td>ACCCTAGAGATCTCAGGTCCTCCAGGATACAA*</td>
<td>MluI/XhoI</td>
<td>AJ224149</td>
</tr>
<tr>
<td>GM-CSF (3286 bp)</td>
<td>CTCGAGGGGCTACGTGGCAAGAAGGCTCTTA*b</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a underline: restriction enzyme recognition sequence

*a Sense primer sequence

*b Anti-sense primer sequence
Herbal extracts preparation and reagents

All the medicinal herbs used in this study have been reported, reputed or claimed to confer immunomodulatory activities in traditional or folk medicine cultures. The tubers of the **Dioscorea batatas**, a traditional Chinese medicinal plant, were air dried, ground into powder, and extracted by water followed by 50% ethanol. The resultant pellet was designated as **D. batatas** fraction I (DsCE-I) and supernatant was again extracted by 75% ethanol to obtain DsCE-II fraction. Air dried aerial parts of **Bidens pilosa** were ground into powder and extracted by hot-water, the solvent was removed in a vacuum and the resultant material was used as a crude extract. Total crude extract of **Echinacea purpurea** whole plants was prepared using 70% ethanol (v/v) extraction. DsCE-I and DsCE-II were normalized by their sugar (mannose) contents (unpublished results and communication from Dr. Shih-Hsiung Wu, Institute of Biological Chemistry, Academia Sinica, Taiwan).

Plant extracts from **B. pilosa** and **E. purpurea** were defined by their specific metabolite profile or index compounds determined using HPLC and various spectral analyses [22, 23]. The normalized and standardized extract preparations were employed for routine promoter activity assays designed for this study. Shikonin, an anti-inflammatory phytocompound of **Lithospermum erythrorhizon** was obtained commercially (TCI, Tokyo, Japan). The RubiDerm cream (Marco Polo Technologies, MD) was used as a commercially available paste containing plant extracts including shikonin. Croton oil, an inflammation inducer, was purchased from Fluka, WI; and Getason cream containing Gentamycin and beta-methasone was obtained from a local pharmacy.

In vivo particle-mediated gene transfer

The Helios gene gun system (Bio-Rad, Hercules, CA) was employed to deliver plasmid DNA into mouse skin per manufacturer’s instructions. In brief, plasmid DNA was precipitated onto gold particles of 2 μm in size (Degussa Corporation, Plainfield, NJ) at a loading rate of 2.5 μg DNA/mg gold particles, and coated onto the inner surface of Tefzel tubing. Each half-inch segment of tubing conferred the delivery of 0.5 mg of gold and 625 ng of plasmid DNA/transfection. Clippers and a mild depilatory treatment were used to remove animal hair from the abdominal area of test mice one day prior to gene-gun bombardment. Mouse skin (3.8 cm²/site) was transfected with test promoter/reporter gene constructs by using a discharge pressure of 350 psi helium. The commercially available drugs, herbal creams, or test herbal extracts were topically applied onto mouse skin tissue as indicated. Normal skin (negative control, without any treatment) and test gene transfected skin treated with or without solvent were used as controls.

Luciferase activity assay

Transfected skin tissues were collected by excision and frozen in liquid nitrogen. Immediately prior to performing the luciferase assay, frozen tissue samples were thawed and scissor minced in 0.5 ml lysis buffer (1% Triton X-100 and a protease inhibitor cocktail in PBS) [9]. Skin tissue lysates were centrifuged at 15,000 rpm for 10 min at 4°C, and the supernatant collected. Transgenic luciferase activity in cell lysate was determined using a luminometer assay (Promega). The levels of luminescent intensities as relative light units (RLU) were translated into picograms of luciferase protein per transfected tissue site, using a standard curve for pure luciferase enzyme assayed in parallel. Results shown represent the mean of three independent transfections.

X-Gal assay

Histochemical staining of β-galactosidase (β-gal) was performed as previously described [9, 24] with some modifications. X-gal substrate was prepared at a final concentration of 40 mg/ml in DMF stock solution. Working solution was made by adding stock X-gal substrate to assay buffer (44 mM HEPES, 3 mM K-ferricyanide, 3 mM K⁺ ferrocyanide, 15 mM NaCl and 1.3 mM MgCl₂, pH 7.4) to make a final 1 mg/ml solution. The excised mouse skin specimen was stretched out on the surface of a wax layer in a 60-mm dish using needles. Six milliliters of X-gal buffer solution was added to each dish and incubated for 2 h at 37°C. The skin tissues were observed under dissection microscopy.

Electrophoretic mobility shift assay (EMSA)

Nuclear protein from mouse skin was isolated as described previously [25]. EMSA was performed using a LightShift Chemiluminescent EMSA kit (Pierce, Rockford, IL) according to the manufacturer’s protocol. A biotin-labeled 30 bp double-stranded oligonucleotide corresponding to the -32 to –3 nucleotide core promoter element of human GM-CSF promoter, 5’- CCTCTGTGTATTTAAGAGCTC TTTTGCCAG-3’ was used as a probe to study the binding of nuclear proteins from untreated and shikonin treated skin samples.

Results

Establishing the in vivo hGM-CSF gene promoter-based assay system

In order to develop a system that can be used to evaluate the skin immune responses to topically applied agents,
pGM620 plasmid containing proximal human GM-CSF promoter fragment (620-bp) was gene gun-transfected into mouse abdominal skin. The basal transgenic promoter activity of pGM620 was 20.0 ± 10.5 pg luciferase protein per blasted skin tissue site (Fig. 1a); in comparison negligible luciferase activity was detected in skin tissue blasted with the promoter-less pGL-3 negative vector (data not shown). To compare the transcriptional activity of the hGM-CSF promoter in pre-inflamed skin or recently inflamed skin, croton oil (2%) was applied to test skin tissue 4 h before or immediately after pGM620 DNA was gene gun-transfected. Skin samples were harvested 8 h post transfection and luciferase activity assayed. Both pretreatment and post-treatment with croton oil enhanced the transcriptional activity of transgenic pGM620 promoter (5.1-fold and 6.4-fold, respectively) compared to solvent treatment and post-treatment with croton oil enhanced the post transfection and luciferase activity assayed. Both pre-gene gun-transfected. Skin samples were harvested 8 h inflamed skin, croton oil (2%) was applied to test skin tissue 4 h before or immediately after pGM620 DNA was induced by topical application of 2% croton oil 4 h before or immediately after bombardment. The treatments were None/GM-CSF: no treatment of blasted skin; Ace/GM-CSF and Cro/GM-CSF: skin treated with acetone (Ace), a solvent base for croton oil (Cro) before gene bombardment; GM-CSF/Ace and GM-CSF/Cro: skin bombarded before acetone or croton oil treatment. Bombarded and treated skin tissues were harvested, extracted and assayed for luciferase activity 8 h after bombardment. (b) Comparison of promoter activities of the extended length (3,286 bp) and the proximal region (620 bp) of the hGM-CSF promoter. The pGL-3-basic, pGM3286 and pGM620 expression plasmids were particle-delivered into test mouse skin, transfected skin tissue sites were then immediately treated with or without 2% croton oil, and skin tissues collected at 16 h post-bombardment and assayed for luciferase activity. Relative luciferase activities (RLA, in arbitrary units) were obtained as comparable luciferase equivalents using standardized, normalized Luminometer assays. Transgenic luciferase activities obtained for 8 h versus 16 h post-bombardment gave similar levels of activities. Data represents the mean ± sd of triplicate skin tissue samples. Results are from one representative experiment; two other independent experiments showed similar results.

Fig. 1 Establishment of an in vivo, inflammation-responsive, transgenic hGM-CSF promoter activity assay. (a) Determination of basal level and inducible transcriptional activity of human GM-CSF gene promoter in mouse skin. pGM620 plasmid was transfected into BALB/c mouse abdominal skin by bombardment with a gene gun, and inflammation was induced by topical application of 2% croton oil 4 h before or immediately after bombardment. The treatments were None/GM-CSF: no treatment of blasted skin; Ace/GM-CSF and Cro/GM-CSF: skin treated with acetone (Ace), a solvent base for croton oil (Cro) before gene bombardment; GM-CSF/Ace and GM-CSF/Cro: skin bombarded before acetone or croton oil treatment. Bombarded and treated skin tissues were harvested, extracted and assayed for luciferase activity 8 h after bombardment. (b) Comparison of promoter activities of the extended length (3,286 bp) and the proximal region (620 bp) of the hGM-CSF promoter. The pGL-3-basic, pGM3286 and pGM620 expression plasmids were particle-delivered into test mouse skin, transfected skin tissue sites were then immediately treated with or without 2% croton oil, and skin tissues collected at 16 h post-bombardment and assayed for luciferase activity. Relative luciferase activities (RLA, in arbitrary units) were obtained as comparable luciferase equivalents using standardized, normalized Luminometer assays. Transgenic luciferase activities obtained for 8 h versus 16 h post-bombardment gave similar levels of activities. Data represents the mean ± sd of triplicate skin tissue samples. Results are from one representative experiment; two other independent experiments showed similar results.

transcriptional activity of pGM3286. Because of the stronger basal level and inflammation-inducible promoter activity of pGM620, we employed this construct in our
assay system to evaluate the enhancing or inhibitory effects of phytocompounds on GM-CSF promoter activity.

In situ analysis of hGM-CSF promoter activity in croton oil-inflamed skin

Our results demonstrated that transgenic hGM-CSF promoter activity could be effectively induced in croton oil-inflamed skin tissue. We then addressed the issue of whether croton oil treatment had resulted in an increase in transfection efficiency (e.g., an increase in the cell number of transgene-expressing cells), or whether it had directly enhanced the level of transgene expression in each transfected cell. To distinguish between these two possibilities, X-gal histochemical staining of transgenic β-galactosidase activity were performed at the tissue and cellular level of test skins. The pGM620-β-gal plasmid and pβ-Gal basic control vectors were gene gun-delivered into mouse skin, and the skin was then inflamed using croton oil. Treated skin tissues were collected at 8 h post-gene gun bombardment and assayed by X-gal staining. No blue-stained positive colonies were observed in skin blasted by the promoter-less pβ-gal basic vector (data not shown). In contrast, β-gal expression driven by pGM620 in transfected skin was enhanced as a bluish ring structure at the tissue level in the inflamed skin, as compared to the normal skin (Fig. 2, 10× magnification, panel A vs. B). At the cellular level (Fig. 2, 30× magnification, panel C vs. D), a higher X-gal staining intensity was observed for individual cells in the inflamed skin tissue than in the normal tissue. By scoring numbers of individual blue color cells per unit skin area in Fig. 2c and d, 389.7 ± 24.0 cells and 382.0 ± 32.9 cells were observed as β-gal positive cells, respectively. Similar results were observed in cell-scoring from other (n = 5) microscopic fields. Therefore, very similar or equal numbers of skin cells were transfected by gene gun bombardment in normal (non-croton oil treated) and inflamed skin tissues. These results suggest that the action of skin inflammation enhanced the transcriptional activity of the transgenic GM-CSF promoter, rather than increased the efficiency of transgene delivery into targeted mouse epidermal cells.

Fig. 2 X-gal histochemical analysis of the effect of inflammation on the transcriptional activity of transgenic hGM-CSF promoter. The pβ-gal basic and pGM620-β-gal plasmids were individually transfected into BALB/c mouse skin using a gene gun. The blasted skin was then treated with or without 2% croton oil, as inflamed or normal skin tissues, respectively. Eight hours post-bombardment the β-galactosidase (β-gal) expressing cells in test skin tissues were examined by X-gal staining. Photomicrographs show β-gal expression for pGM620-β-gal in normal skin tissues (a and c) and inflamed skin tissues (b and d). No blue-stained cells were observed in skin tissues that were blasted with pβ-gal basic (vector only) plasmid (data not shown). Results are representative of one experiment, other independent experiments showed similar results.
Effect of medicinal plant extracts and derived phytocompounds on transcriptional activity of hGM-CSF promoter in normal or inflamed skin tissues

In order to evaluate the use of the present in vivo transgenic promoter activity assay for assessing immunomodulatory activities, plant extracts from Bidens pilosa, E. purpurea and D. batatas, were topically applied (at 10 μg/10 μl per tissue site) to pGM620-blasted skin tissue. DsCE-I, a 50% ethanol partitioned fraction of the D. batatas aqueous extract, induced a substantial increase (1.8-fold higher) in pGM620 promoter activity (239.7 ± 19.2 RLA/blasted skin site) than the water treated vehicle control (133 ± 27.3 RLA/site), suggesting a modest pro-inflammatory effect. In comparison, no significant enhancement or suppression of promoter activity was observed with other herbal extracts tested (Fig. 3a). These results therefore show that the transgenic hGM-CSF promoter assay in skin could selectively and specifically respond to different medicinal plant extracts.

To investigate whether the pGM620 promoter assay would respond to anti-inflammatory agents, the effect of selected phytocompounds and medicinal plant extracts on pGM620 promoter activity was examined in croton oil-inflamed skin tissues. pGM620 was gene gun transfected into mouse skin, and test epidermal tissues treated immediately with 2% croton oil for 1 h to induce inflammation. One hour after inflammation induction, herbal extracts, single phytocompounds, and the commercial drugs Getason and RubiDerm were applied individually onto inflamed skin sites. Skin tissues inflamed with croton oil but not treated with test agents were used as controls. As shown in Fig. 3b, DsCE-I induced a significant 1.8-fold increase in transcriptional activity of pGM620 over the control. A marginal increase (∼27%) in promoter activity was also observed by treatment with B. pilosa plant extract. In contrast, shikonin significantly suppressed pGMG620 promoter activity to 11.6% of the control value (100%). RubiDerm and Getason inhibited pGM620 promoter activity to 67% and 40%, respectively, of the control values. These results suggest that the current assay system could effectively discriminate between phytocompounds with potential pro- versus anti-inflammatory bioactivities.

Evaluation of transcriptional activities of other pro-inflammatory cytokine gene promoters in skin tissue

Evidence has shown that dermatitic inflammatory reactions are associated with the activation of a group of specific cytokine genes [21, 27]. In order to test the response of other pro-inflammatory cytokine gene promoters in skin tissues, three human cytokine gene (IL-1β, IL-18, and TNF-α) promoters were PCR-amplified and cloned into pGL-3 basic vector. These plasmid constructs were

![Fig. 3](image_url)

**Fig. 3** Effects of phytocompounds or plant extracts on the transcriptional activity of the hGM-CSF promoter in mouse skin tissue. (a) Normal skin. Fold-changes in luciferase activity were obtained by comparing the values of phytocompound-treated test samples over the water-treated (vehicle control) sample. pGM620 plasmid was transfected into BALB/c skin tissue, and 1 h later water or herbal extracts prepared in water were applied to blasted skin at 10 μg/10 μl/tissue site. Test skin tissues were harvested for assay of transgenic luciferase activity/protein levels at 8 h post-treatment. Water: 10 μl/site; B. pilosa hot water extract; E. purpurea 70% ethanolic extract; D. batatas 70% ethanolic extract; DsCE-I and DsCE-II: D. batatas water extract fraction as described in “Materials and Methods”; and D. batatas hot water extract. Data represent the results from triplicate skin samples. Another experiment showed similar results. Significant increase is indicated by * with a p value <0.05. (b) Inflamed skin. Fold change in luciferase activity is the same as that defined above. pGM620 plasmid was transfected into BALB/c mouse skin tissue and the skin was inflamed with 2% croton oil as described in the legend of Fig. 2. An hour later, test herbal extracts or commercial medicines were applied onto inflamed or normal (untreated, control) skin. Test skin tissues were harvested for transgenic luciferase activity assay 8 h post treatment with test herbal extract or medicine. Test substances and applied doses were as follows: water 10 μl/site; saline 10 μl/site; B. pilosa hot water extract (100 μg/site); DsCE-I: D. batatas water extract fraction I (100 μg/site); Shikonin: 10 μg/site; RubiDerm (5.4 mg/site); and Getason: β-methasone (54 μg/site). Data represents the mean ± sd of triplicate tissue samples. Results are representative of one experiment. Two other independent experiments showed similar results. Significant inhibition is indicated by ** with a p value <0.01, Student’s t-test.
individually blasted into mouse skin and left untreated or immediately treated with 2% croton oil to induce inflammation. Under normal skin conditions, the three tested promoters revealed a wide range of basal level transcrip-
tional activity, as detected at 8 h post-transfection. The TNF-
\( \alpha \) promoter showed the highest level of transgenic luciferase protein (326.3 ± 23.5 RLA/blasted skin site) expression. In contrast, the promoters of IL-1\( \beta \) and IL-18 conferred 46.9 ± 10.7 RLA and 32.3 ± 8.0 RLA of transgenic luciferase proteins per blasted skin site, respectively. Upon inflammation of skin tissue, the promoter activities of TNF-\( \alpha \), IL-1\( \beta \) and IL-18 increased 3.4-,
1.5- and 1.7-fold, respectively (Fig. 4). TNF-\( \alpha \) promoter was also sensitive to croton oil-induced inflammation in a similar way to GM-CSF promoter (5 to 6-fold increase) (Fig. 4). TNF-\( \alpha \) promoter activity was routinely found to be increased 3 to 4-fold (data not shown). Our data suggests that the current transgenic assay could be effectively employed to profile various cytokine promoter activities in vivo and in situ in response to inflammation.

Molecular basis for shikonin inhibition of GM-CSF promoter activity in skin tissue

Our results show that shikonin can significantly suppress pGM620 promoter activity caused by croton-oil induced inflammation. We have previously reported that shikonin suppresses the transcription of TNF-\( \alpha \) by inhibiting the binding of TFIID protein complex (TBP) to TATA box in the GM-CSF promoter [25]. We thus conducted an EMSA study to determine whether shikonin suppresses GM-CSF promoter activity through interfering with TBP binding activity. Mouse skin was transfected with pGM3286 DNA and topically untreated or treated with solvent alone, or treated with a dose of 1, 10, or 100 µg per site of shikonin. Skin samples were harvested after 1 h and nuclear extracts were subjected to EMSA analysis as described in “Materials and Methods”. Binding specificity of the complexes (lanes 2–7) was analyzed by competition against the GM-CSF probe using 1000-fold excess of unlabeled DNA probe (lane 3).

Discussion

It is well known that cytokine expression and function is a complex process. Factors affecting the process include the
time of release of specific cytokines, the local environment in which the cytokines act, the presence of antagonistic or synergistic molecules, the density of cytokine receptors on cells, and interactions with other cytokines. Consequently, experimental studies on cytokine biology and related cellular immunology are challenging and sometimes controversial. To measure the expression level of cytokine genes in vivo, Northern blot, RT-PCR, ELISA, and immunohistochemistry are currently employed; however, these techniques are relatively time-consuming and labor intensive. This study describes the establishment of an in vivo, promoter activity-based assay system that can effectively measure relative transgenic cytokine gene promoter activities in response to pro- or anti-inflammatory stimuli and to specific phytocompounds. This in vivo gene assay system may have broad medicinal and biotechnological applications.

As seen in Fig. 2, we have demonstrated here that our present epidermal keratinocyte system can be effectively visualized and employed at the cellular and tissue levels for molecular biology and cell research studies of the immune system of the skin under in vivo physiological or pathological conditions. Future experiments using confocal microscopy are also expected to reveal sub-cellular or organelle structures or features that are involved in various skin inflammations under in vivo conditions.

Temporal and adjustable boosts to the immune system are considered to be key therapeutic strategies. GM-CSF has been considered as an adjuvant strategy to improve the efficacy of candidate DNA cancer vaccines [16, 28]. The results of our study showed that croton oil elevates GM-CSF promoter activity in transfected skin cells (Fig. 1) and in in situ β-gal activity-staining analyses (Fig. 2). This result is similar to a previous report [29] that showed that croton oil can induce the expression of GM-CSF in keratinocytes and mouse skin. Our findings suggest that phytocompounds exhibiting analogous proinflammatory function to croton oil (which is quite toxic) may have the potential to be developed as topically applied adjuvants for immunotherapies or cancer vaccines. Taking this into account, we showed that DsCE-I (a fraction of D. batatas tuber extract) enhanced GM-CSF promoter activity in both normal (Fig. 3a) and inflamed skin (Fig. 3b). Inflammation is a crucial physiological reaction to infection and tissue damage induced by physical and chemical factors. GM-CSF has been considered to play a central role in maintaining chronic inflammation [20]. Our results (Fig. 3b) demonstrated that β-methasone (e.g., as Getason) decreased the transcriptional activity of GM-CSF promoter in croton oil-inflamed skin. This result agrees with a previous study showing the suppression of GM-CSF expression by glucocorticoids [30]. Shikonin and Rubi-Derm inhibited the activity of the transgenic GM-CSF promoter, whereas extract of B. pilosa showed no effect, furthermore a fraction of D. batatas (DsCE-I) increased the activity of GM-CSF promoter. Taken together, our results demonstrate that this in vivo assay system can effectively delineate both the activation and inhibition effects of test materials on GM-CSF gene promoter.

Inflammation is involved in a spectrum of common skin disorders, both intrinsic chronic disorders and extrinsic, chemical or physical damage. Previous studies [31–33] have shown that different types of skin inflammation may be associated with the activation of different specific groups of cytokines. For example, the chronic inflammatory skin disease atopic dermatitis, as compared with psoriasis, was found to be associated with elevated Th2 cytokine (IL-4 and IL-13) expression and low levels of pro-inflammatory cytokines such as TNF-α, IFN-γ, and IL-1β [31] in skin. Irritant and allergic contact dermatitis are the two most frequent manifestations of skin toxicity [32, 33]. Clinically, it is difficult to distinguish these two types of dermatitis, but studies have suggested that both irritants and allergens can activate TNF-α and GM-CSF [21]; while only contact allergens up-regulate IL-α and IL-1β [21, 34]. We therefore evaluated whether an alternative strategy, the current transgene assay system, could differentiate between the cytokine expression profiles of these two types of dermatitis. Croton oil is a known irritant that stimulates keratinocytes to release inflammatory mediators such as IL-1α, TNF-α, IL-8, and GM-CSF [26, 29]. Our assay system (Fig. 4) shows that croton oil can significantly up-regulate GM-CSF and TNF-α promoter activities while only slightly enhancing IL-1β and IL-18 promoters in skin. These results suggest that it may be possible to apply the current transgene assay system to the systematic analysis of cytokine expression profiles in skin or other epithelial tissues.

In the present study we showed that shikonin, an anti-inflammatory phytocompound, suppressed human GM-CSF promoter activity by inhibiting TFIID (TBP) binding to TATA box. This result is consistent with our previous studies on TNF-α [25]. We suggest that shikonin may act through a common molecular signaling pathway(s) to exert its inhibitory role on the transcriptional regulation of a set of functionally related cytokine genes. Future studies are needed to determine how different cytokine genes are differentially affected by shikonin and other anti-inflammatory compounds.

This study provides an in vivo three-dimensional tissue assay system that has an advantage over the two-dimensional, (e.g., in vitro) cell culture systems. The findings of this in vivo study provide useful information on the application of phytocompounds or other agents as topical immunomodulators on skin. This experimental approach may also be employed to explore the underlying
mechanisms of various cytokines or chemokines actively involved in various immunological activities in skin.

Acknowledgements This work was supported by research grants of No. 91S701911 and No. 94S-0402 from the National Science and Technology Program for Agricultural Biotechnology, and an institutional grant from Academia Sinica, Taiwan, R.O.C.

References