



Stimulation of Langerhans cells with ketoprofen plus UVA in murine photocontact dermatitis to ketoprofen

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Summary

Background: Ketoprofen (KP) clinically evokes the allergic type of photocontact dermatitis when applied to the skin and irradiated with ultraviolet A (UVA). We have established a murine model of photocontact dermatitis to KP, which is a T cell-mediated delayed type hypersensitivity.

Objective: To further explore the mechanism underlying this sensitivity, we investigated whether KP plus UVA activates the antigen-presenting ability of Langerhans cells (LCs).

Methods: We analyzed the expression of surface molecules on LCs in the murine epidermis treated with KP plus UVA by immunohistochemistry and flow cytometry. Changes in the cytokine expression of epidermal cells from KP-phototreated skin were also examined by real-time PCR.

Results: LCs became larger after treatment with KP plus UVA. The number of LCs was significantly decreased 2–3 days after KP phototreatment and recovered on day 5. A flow cytometric analysis revealed that KP plus UVA increased the percentage of LCs that highly expressed MHC class II, CD86, CD80, CD54 and CD40, whereas neither KP nor UVA alone enhanced the expression. KP phototreatment augmented the expression of I-A and CD86 on LCs in KP and UVA dose-dependent manners. A real-time PCR analysis of KP-phototreated skin showed that the expression of mRNA for IL-1 α and GM-CSF was immediately increased after treatment.

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Conclusion: A photosensitizing regimen of KP plus UVA activates LCs at least partly by stimulating keratinocytes to produce cytokines. Two strains of mice (BALB/c and AKR) differ in responsiveness to KP and the difference is not related to the activation of keratinocytes.

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1. Introduction

Ketoprofen (KP), an arylpropionic acid derivative widely used as a topical anti-inflammatory drug, is clinically well known to induce photocontact dermatitis as an adverse reaction, and ultraviolet A (UVA) is the action spectrum of this photosensitivity [1–7]. In general, photoreactions by photosensitive chemicals are divided into the phototoxic and photoallergic types. While phototoxicity is mediated by active oxygen, especially singlet oxygen [8,9], photoallergy occurs as a consequence of a specific immune reaction mediated by antigen-specific, sensitized T cells [10–14]. KP has both phototoxic and photoallergic potentials, but many clinical observations have indicated that photosensitivity to KP is a photoallergic reaction [1–7].

The two theories, named photohaptens and prohaptens models, have been put forward to explain the formation of photoallergen [11,14]. According to the photohaptens theory, photosensitizing chemicals and protein need to coexist upon exposure to UVA in a noncovalent manner, and UVA turns it covalent [11,14]. On the other hand, the prohaptens theory suggests that UVA simply converts photosensitizing substances into ordinary haptens, which subsequently binds to protein. It seems that most of photoallergic substances have a photohaptenic moiety, as typically demonstrated in 3,3',4',5-tetrachlorosalicylanilide (TCSA) [15–18], quinolones [10–12], and afloqualone [13]. KP serves as a photohaptens because of its photocoupling ability to protein [19]. Our study has suggested that photohaptens bind to self-peptides located in the groove of major histocompatibility complex (MHC) molecules of antigen-presenting cells [20].

In photocontact dermatitis, epidermal Langerhans cells (LCs) serve as photoantigen-presenting cells and transport a given photoantigen to the draining lymph nodes where they present it to photoantigen-specific T cells. Upon stimulation with an ordinary haptens, LCs are matured to highly express MHC class II molecules [21]. Not only MHC class II molecules but also costimulatory molecules such as CD86 and CD80 and adhesion molecules such as CD54 and CD40 play in important roles on the antigen-presentation [22–25]. Likewise, representative photohaptens TCSA [26,27] plus UVA augments

the expression of MHC class II, CD86, CD80 and CD54 on LCs [16]. It is well known that keratinocyte-derived granulocyte/macrophage colony stimulating factor (GM-CSF), interleukin (IL)-1 α and tumor necrosis factor (TNF)- α are essential for the viability of LCs, and upregulate the antigen-presenting activity of LCs [23,28–30]. Thus, it is possible that photohaptens plus UVA exerts the LC-stimulating action by stimulating keratinocytes to produce these cytokines.

We have previously established a murine model of photocontact dermatitis to KP [31]. Mice bearing H-2^k such as AKR are high responders, whereas H-2^d mice such as BALB/c mice are low responders in this sensitivity [31]. This is in contrast to photocontact dermatitis to TCSA, in which H-2^d and H-2^k mice are high and low responders, respectively [15,17]. KP photosensitivity is mediated by T cells, and both CD4⁺ and CD8⁺ T cells are required for the full blown sensitivity [31]. Th2 cytokines/chemokines as well as Th1 cytokines/chemokines are expressed in the lymph nodes and epidermis of challenged mice. In this study, we investigated alterations in the morphology, cell number, and surface molecule expression of LCs treated with KP plus UVA. Keratinocyte-derived LC-supportive cytokines were also examined. Results suggest that KP plus UVA play an immunostimulatory role for these cells in the mouse strain highly responsive to KP phototreatment.

2. Materials and methods

2.1. Chemicals and monoclonal antibodies (mAbs)

Ketoprofen (KP) was obtained from Hisamitsu Pharmaceutical Co., Inc. (Tokyo, Japan) and TCSA was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Anti-Fc γ II/III receptor (2.4G2) monoclonal antibody (mAb), phycoerythrin (PE)-labeled anti-mouse I-A^d (AMS-32.1) and I-A^k (11–5.2) mAbs, and fluorescein isothiocyanate (FITC)-labeled anti-mouse CD86 (GL1), CD80 (16-10A1), CD54 (3E2), CD40 (3/23) mAbs and isotype matched immunoglobulins (Igs) were purchased from BD Pharmingen (San Diego, CA).

2.2. Mice

AKR (H-2^k) and BALB/c (H-2^d) mice were obtained from Kyudo Co., Ltd. (Kumamoto, Japan). Female mice, 8–10 weeks old, were used unless otherwise mentioned.

2.3. Treatment of earlobes with KP plus UVA

As UVA source, black light (FL20SBL-B) emitting UVA ranging from 320 to 400 nm with a peak emission at 365 nm was purchased from Toshiba Electric Co. (Tokyo, Japan). With a UV radiometer (Topcon Technohouse Corp., Tokyo, Japan), the energy output of three 20-W tubes of black light at a distance of 20 cm was 1.8 mW/cm² at 365 nm. Irradiation was performed through a pane of 3 mm thickness glass. Both sides of each earlobe of mice were painted with 10 μ l of KP in ethanol and irradiated with UVA. Unless otherwise mentioned, 20 or 10% KP and 32 J/cm² UVA were used.

2.4. LC enumeration in epidermal sheets

Epidermal sheets were separated from dermis with 0.5 M ammonium thiocyanate, fixed in acetone for 5 min at -20°C , and stained with biotinated anti-I-A^d mAb at a final concentration of 4 μ g/ml for 18 h at 4°C , and then with Cy3-streptavidin at a final concentration of 2 μ g/ml for 2 h at 37°C . Stained LCs were enumerated in two random visual field at 200 $\times$ magnification in two different sections and were expressed as cells/mm².

2.5. Preparation of epidermal cell suspensions and enrichment of LCs

The procedure was previously described [12,32]. Briefly, cartilage-removed skin sheets from earlobes were floated in 0.2% trypsin in phosphate buffered saline (PBS; pH 7.4) for 1 h at 37°C . Epidermis was then separated from dermis with forceps in PBS supplemented with 10% fetal calf serum (FCS). Epidermal cell suspensions were prepared by vigorous pipetting and filtration through nylon mesh. For enrichment of LCs, freshly isolated single cell suspensions of epidermal cells were centrifuged over a Ficoll-Hypaque gradient and interface cells were collected.

2.6. Flow cytometry

All mAbs were used at 1–5 μ g/10⁶ cells, and each incubation was performed for 30 min at 4°C , followed by two washes in PBS supplemented with 5%

FCS and 0.02% sodium azide. LC-enriched epidermal cells were preincubated with 2.4G2 for 5 min to prevent non-specific binding of the subsequent reagents to Fc receptors and stained with anti-I-A^k mAb in AKR mice or anti-I-A^d mAb in BALB/c mice and anti-CD86, -CD80, -CD54 or -CD40 mAb. Fluorescence profiles were analyzed in a FACScalibur (Becton Dickinson, Franklin Lakes, NJ). LCs were identified with the expression of MHC class II and cell size. Viable cells were identified by 7-AAD uptake. The mean fluorescence intensity (MFI) was calculated by using CellQuest, which is an operating software for a FACScalibur. The relative MFI was calculated with the ratio to each control.

2.7. Quantitative RT-PCR

Total mRNA was extracted from epidermal cell suspensions with the SVTotal RNA Isolation system (Promega, Madison, WI) according to the manufacturer's protocol. Target gene expression was quantified in a two-step RT-PCR. cDNA was reverse transcribed from total RNA samples using the TaqMan RT reagents (Applied Biosystems, Foster City, CA). Murine IL-1 α (Assay ID: Mm00439620_m1) and GM-CSF (Assay ID: Mm00438328_m1) expression was quantified using TaqMan Gene Expression Assay (Applied Biosystems) in the ABI PRISM 7000 sequence detection system (Applied Biosystems). As an endogenous reference for these PCR quantification studies, GAPDH gene expression was measured using the TaqMan rodent GAPDH control reagents (Applied Biosystems). The relative expression was calculated using the $2^{-\Delta\Delta C_T}$ method [33]. The expression of the target gene normalized to an endogenous reference and relative to calibrator is given by the formula $2^{-\Delta\Delta C_T}$. Gene expression in untreated mice was used as a calibrator expression to calculate $\Delta\Delta C_T$.

2.8. Photocontact dermatitis to KP and TCSA

The procedures for induction and elicitation of photocontact dermatitis to KP [31] and TCSA [15] were reported previously. Briefly, for KP photosensitivity, mice were sensitized on days 0 and 1 by painting with 50 μ l of 4% KP in acetone and subsequent irradiation with 20 J/cm² UVA on the shaved abdomen, and challenged on day 5 with 25 μ l of 2% KP in ethanol plus 20 J/cm² UVA on each earlobe. For TCSA photosensitivity, mice were sensitized with 50 μ l of 1% TCSA in acetone plus 12 J/cm² UVA on day 0 and 1 and challenged on day 5 with 20 μ l of 0.1% TCSA in ethanol plus 20 J/cm² UVA. The ear swelling responses were measured before and after

sensitization with a dial thickness gauge. Data were expressed as Δ ear thickness.

2.9. Statistical analysis

The *P* values were calculated using Dunnett type multiple comparison, Student's *t*-test or Paired *t*-test. Values ≤ 0.05 were considered significant.

3. Results

3.1. Morphological and numerical changes of LCs in KP-phototreated skin

The dorsal aspect of murine earlobes were painted with 5 μ l of 20% KP in ethanol and irradiated with 32 J/cm² UVA. Epidermal sheets were obtained from earlobes 1, 2, 3 or 5 days after KP phototreatment and stained with anti-I-A^d mAb. As typically observed on day 2, LCs in the epidermis treated with KP plus UVA were stained brightly for MHC class II molecules (Fig. 1). They became larger and maintained well-developed dendritic configuration. This morphological alteration was virtually the same as that seen in LCs phototreated with TCSA, a representative photohaptan [16]. There was no change in

LCs treated with KP or UVA alone. Thus, both KP and UVA were necessary for stimulation of LCs.

The numerical change was assessed in the epidermal sheets. When earlobes were phototreated with KP at 20%, the number of MHC class II⁺ cells was unchanged on day 1, significantly decreased on days 2 and 3, and recovered on day 5 (Fig. 2). The comparable change was observed in LCs phototreated with 0.2% TCSA.

3.2. Enhanced expression of MHC class II and other surface molecules on LCs by KP phototreatment

The expression levels of MHC class II and costimulatory molecules on LCs were examined in earlobes painted with 10 μ l of 10% KP in ethanol and irradiated with 32 J/cm² UVA. LC-enriched epidermal cell suspensions were prepared from earlobes 18 h after KP phototreatment, and the expression of each molecule was analyzed by flow cytometry. Immature LCs express a high level of MHC class II and low levels of CD86, CD80, CD54 and CD40 molecules. Upon maturation, the expression of all the molecules is upregulated [21–25]. Fig. 3A shows representative data gated for I-A⁺ cells so that only LCs were analyzed. The stimulation level of LCs was

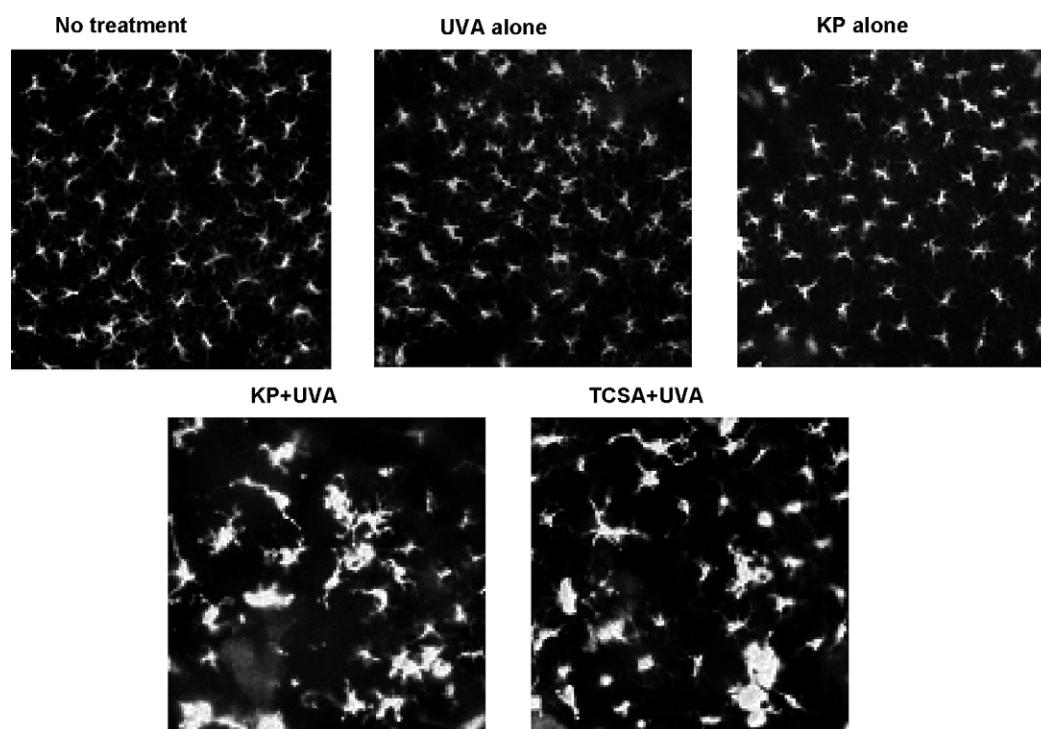


Fig. 1 Morphological change of epidermal MHC class II⁺ LCs from KP-phototreated skin. Epidermal sheet from earlobes non-treated or treated with UVA alone, 20% KP alone, 20% KP plus 32 J/cm² of UVA, or TCSA plus 32 J/cm² of UVA were stained with anti-I-A^d and analyzed by fluorescent microscopy. Data indicate representative images of epidermal I-A^d LC (*n* = 4 or 6). In all photographs, the original magnification is 200 $\times$.

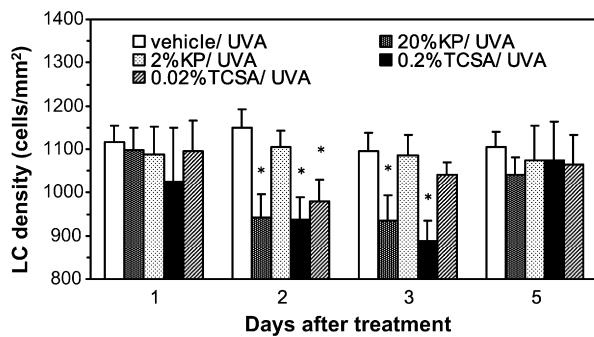


Fig. 2 Decreased density of LCs in epidermis treated with KP plus UVA. Epidermal sheets from earlobes treated with vehicle plus 32 J/cm² of UVA, 20% KP plus 32 J/cm² of UVA, 2% KP plus 32 J/cm² of UVA, 0.2% TCSA plus 32 J/cm² of UVA, or 0.02% TCSA plus 32 J/cm² of UVA were stained with anti-I-A^d and analyzed by fluorescent microscopy. The positively stained LCs were enumerated in two random visual field at 200 $\times$ magnification in two different sections and were expressed as cells/mm². Statistical analysis was performed with Dunnett type multiple comparison. * $p < 0.05$ vs. vehicle plus UVA.

expressed as the percentage of I-A-highly positive and CD86-positive population. LCs from mice untreated, or treated with KP or UVA alone contained no substantial number of the activated cells. Treatment with KP plus UVA increased the percentage of activated LCs, similarly to TCSA phototreatment, but at a lower level. Thus, both KP and UVA were required for the augmentation of MHC class II and CD86 expression on LCs.

In addition to I-A and CD86, CD80, CD40 and CD54 were also examined. The percentages of LCs expressing these molecules were increased by KP plus UVA, but the numbers of positive cells were fewer than that of I-A⁺CD86⁺ cells (Fig. 3B). Again, the levels of upregulation were lower than TCSA phototreatment. Moreover, the data with isotype matched controls indicated no interference by a non-specific binding of Igs in any treatment.

Earlobes of mice were treated with 0.4, 2 or 10% KP in combination with 32 J/cm² UVA (Fig. 4A). Alternatively, earlobes painted with 4% KP were irradiated at 0, 8, 16, 32 or 64 J/cm² of UVA (Fig. 4B). The expression of both MHC class II and CD86 on LCs was elevated in dose-dependent manners up to the amounts monitored.

3.3. Differences in the MHC class II and CD86 expression induced by KP or TCSA phototreatment between AKR and BALB/c mice

As represented by Fig. 5A and B, in murine photocontact dermatitis, AKR mice are high responders

for KP and low responders for TCSA, whereas BALB/c mice are high for TCSA and low for KP [15,31]. In particular, the low responsiveness of AKR mice to TCSA is intriguing. Therefore, we investigated differences in the augmented expression of MHC class II and CD86 by phototreatment with KP or TCSA between AKR and BALB/c mice. The two strains of mice were painted with 10 μ l of 10% KP or 0.2% TCSA in ethanol on earlobes and irradiated with 32 J/cm² of UVA. Although differences between AKR and BALB/c in the expression of MHC class II and CD86 by KP phototreatment was not statistically significant, AKR mice tended to exhibit slightly high expression of these molecules as compared to BALB/c mice in the independent individual experiments. In TCSA phototreatment, BALB/c mice showed significantly higher expression of these molecules than did AKR mice. These results suggested that each photohapten has different photostimulatory ability for LCs, depending on the mouse strain, and the magnitude of photocontact dermatitis is associated with the levels of MHC class II and costimulatory molecules.

3.4. Enhanced expression of IL-1 α and GM-CSF in keratinocytes treated with KP plus UVA

Total RNA was extracted from epidermal cells of earlobes phototreated with KP and subjected to real-time PCR for keratinocyte-derived cytokines, IL-1 α , and GM-CSF. Earlobes were taken from mice immediately (0 h) or 12 h after cessation of UVA irradiation. The relative quantity of GM-CSF was remarkably increased at 0 h and decreased to the untreated level at 12 h (Fig. 6). IL-1 α expression of KP-phototreated skin was upregulated, but to a lesser degree of TCSA phototreatment. Treatment with KP or UVA alone was not stimulatory.

4. Discussion

We have previously shown that photocontact dermatitis to KP is induced in mice and mediated by T cells [31]. Our present study was conducted to investigate the early sensitization event in this sensitivity, focusing on KP-induced alterations in LCs. We demonstrated that KP plus UVA, but not KP or UVA alone, induces morphological changes of epidermal LCs and stimulates them to express MHC class II, CD86, CD80, CD54 and CD40 molecules, indicating that KP phototreatment matures LCs. Moreover, LC density in the epidermal sheets was decreased by KP phototreatment. In our murine model of photocontact dermatitis to KP, we used

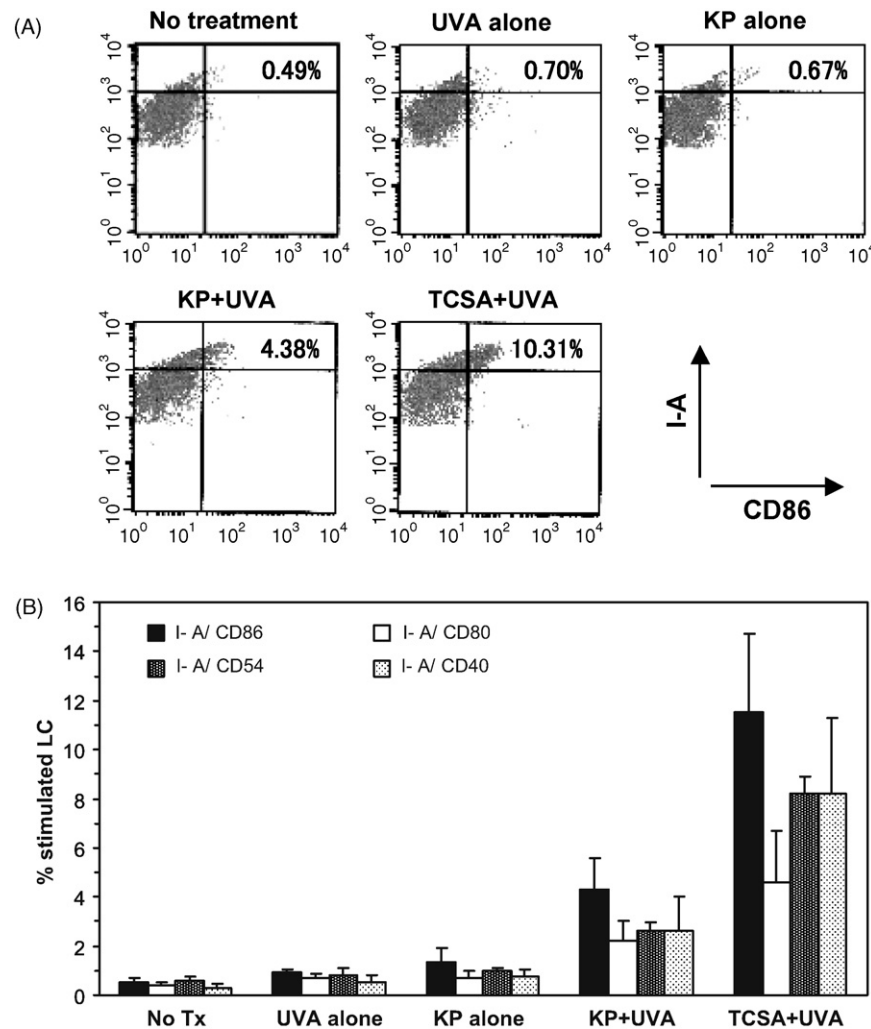


Fig. 3 Augmented expression of MHC class II, CD86, CD80, CD54 and CD40 on LCs in epidermis treated with KP and UVA. LC-enriched epidermal cells from earlobes non-treated, or 32 J/cm² of UVA alone, 10% KP alone, 10% KP plus 32 J/cm² of UVA, or 0.2% TCSA plus 32 J/cm² of UVA were double-stained with anti-I-A^d and anti-CD86, -CD80, -CD54, or -CD40 mAbs and analyzed by flow cytometry. (A) Data indicate representative dot-plots of LCs stained with anti-I-A^d and anti-CD86 mAbs. Percent in each dot-plot indicates the rate of I-A^d⁺ and CD86⁺ stimulated LC. (B) Data indicate mean values of I-A^d⁺ and CD86⁺, CD80⁺, CD54⁺, or CD40⁺ stimulated LCs ($n = 4$ or 5). The error bars represent S.D.

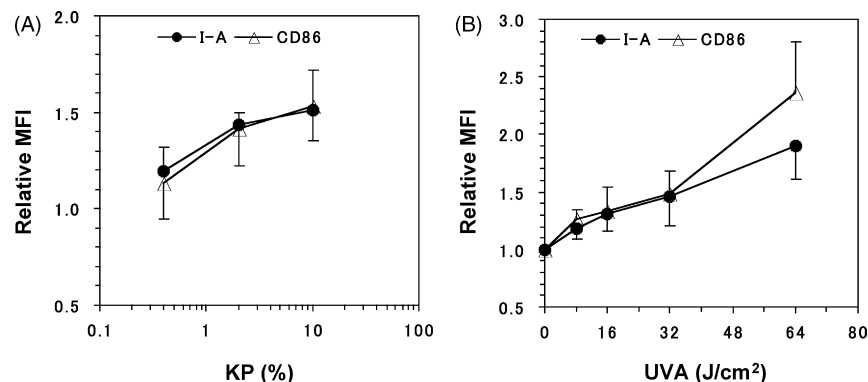


Fig. 4 Dose dependent augmentation of MHC class II and CD86 expression on LCs by KP and UVA. Mice were treated with various concentrations of KP plus 32 J/cm² of UVA (A) or 4% KP plus various doses of UVA (B). LC-enriched epidermal cells from earlobes were double-stained with anti-I-A^d and anti-CD86 mAbs and analyzed by flow cytometry. Data indicate the expression of MHC class II (closed circle) and CD86 (open triangle). Relative MFI values were calculated to individual control of 0% KP plus 32 J/cm² of UVA (A, $n = 3$) or 4% KP plus 0 J/cm² of UVA (B, $n = 3$). The error bars represent S.D.

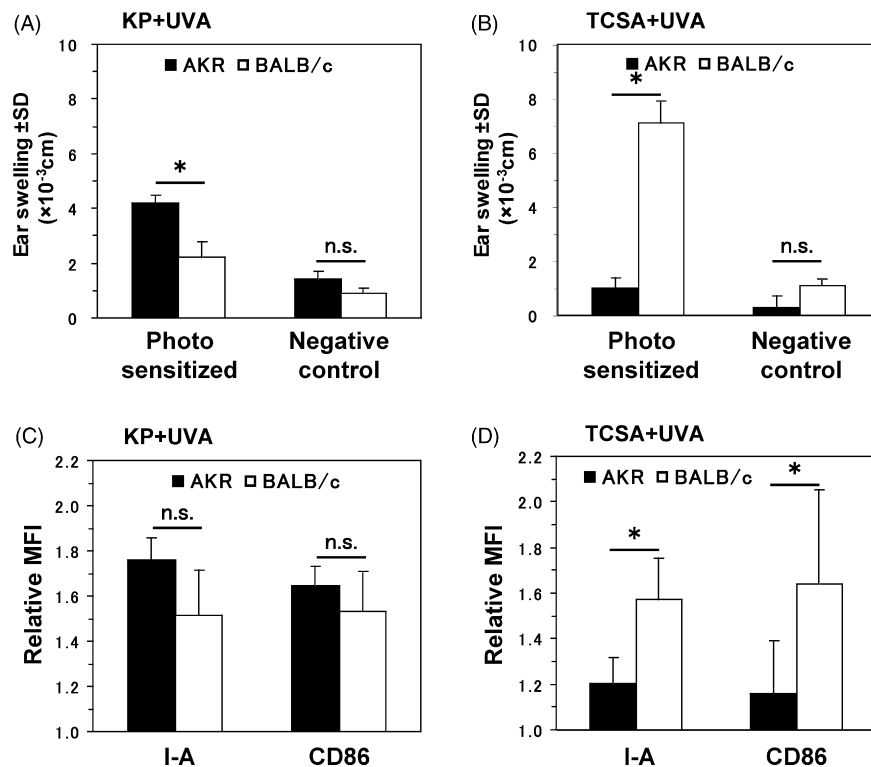


Fig. 5 Comparison between AKR and BALB/c mice in susceptibility of LC expression of MHC class II and CD86 to KP plus UVA. (A and B) AKR (closed column) and BALB/c (open column) mice were photosensitized with 4% KP (A) or 1% TCSA (B) and photochallenged with 2% KP (A) or 0.1% TCSA (B). The ear swelling responses were measured ($n = 4-6$). Statistical analysis was performed with Student's *t*-test. * $p < 0.05$. (C and D) LC-enriched epidermal cells from earlobes non-treated or treated with 10% KP plus 32 J/cm² of UVA (C) or 0.2% TCSA plus 32 J/cm² of UVA (D) were double-stained with anti-I-A^d and anti-CD86 mAbs and analyzed by flow cytometry. Relative MFI values were calculated to individual non-treatment control ($n = 3$). Data indicate augmentation of MHC class II and CD86 expression in AKR (closed column) and BALB/c (open column) mice. The error bars represent S.D. Statistical analysis was performed with Paired *t*-test. * $p < 0.05$.

4% in 50 μ l solution and 2% KP in 25 μ l solution for sensitization and challenge, respectively. In the present LC morphology and number studies, the alterations were obtained with 20%, but not 2%, KP in 5 μ l solution. This raises the possibility that the total applied amount of KP determines the response. Alternatively, some antigen-presenting cells other than LCs, such as dermal dendritic cells, might play a role for sensitization and elicitation of photocontact dermatitis. Compared to ordinary contact hypersensitivity, however, UVA irradiation is required for sensitization and challenge, and therefore, dermal dendritic cells seem to be less photomodified with KP than LCs. The role of such antigen-presenting cells is an issue to be elucidated. There are two possibilities in the reduction of LC number. One is that LCs undergoing maturation migrate to the draining lymph nodes, as previously documented in ordinary haptens [34,35]. The other possibility remains that KP exerts phototoxic action to LCs. Considering that we analyzed only viable LCs with high CD86 expression in our flow cytometry study, the phototoxic effect is

minimal in this reduction, and the migration mechanism is more likely.

The ability of KP phototreatment to mature LCs was not so high as that of TCSA phototreatment. This suggests that KP photoproducts are formed to a lesser degree than TCSA photoproducts, or the photoantigenicity of KP is weaker than TCSA. Alternatively, KP possibly affects LC maturation by its pharmacological action of nonsteroidal anti-inflammatory drug. Contact sensitization of the skin with a hapten stimulates keratinocytes to release prostaglandin E₂ (PGE₂), which promotes LC migration to lymph nodes [36]. We found that KP suppresses the production of PGE₂ by murine keratinocytes (unpublished observation).

It is well known that keratinocytes release many cytokines, some of which play a supportive role for LCs. GM-CSF is essential for maintenance of LC viability and upregulation of LC function [23,28-30]. IL-1 α upregulates LC function in the presence of GM-CSF [29,30]. TNF- α also plays an important role in the viability and migration of LCs [23,30]. In the present study, keratinocytes were stimulated to

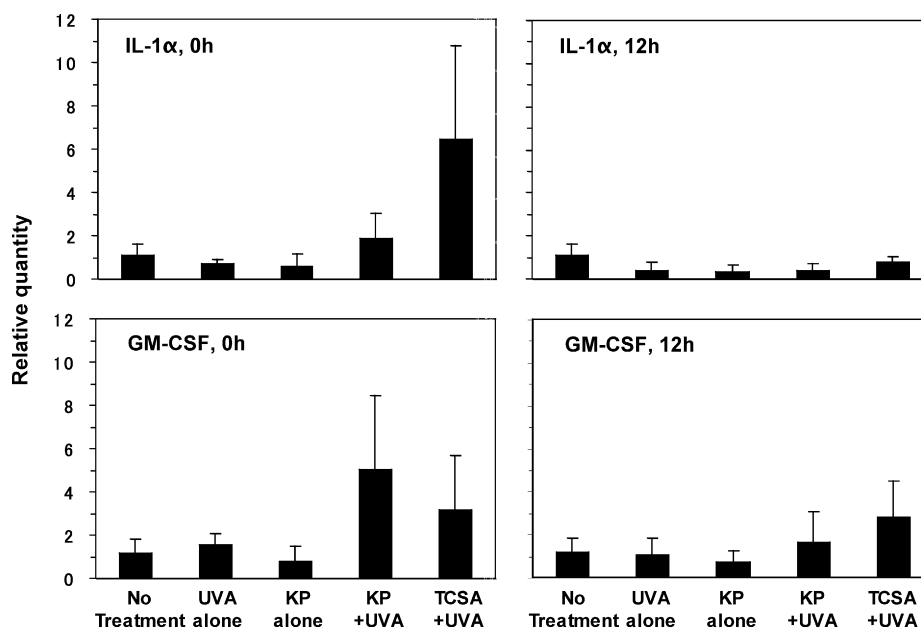


Fig. 6 Augmented expression of cytokine mRNA in epidermal cells. Total mRNA was extracted from epidermal cells from earlobes of non-treated or treated with 32 J/cm² of UVA alone, 10% KP plus 32 J/cm² of UVA, or 0.2% TCSA plus 32 J/cm² of UVA. Murine IL-1 α , GM-CSF and TNF- α gene expression were quantified in a two-step RT-PCR using TaqMan probes. GAPDH gene expression was measured as an endogenous control. Results represent normalized mean target mRNA amounts relative to non-treated mice using the $\Delta\Delta C_T$ method ($n = 4$ or 5). The error bars represent S.D.

produce IL-1 α and GM-CSF by KP plus UVA, suggesting that LC maturation is mediated at least by these keratinocyte-derived cytokines.

In murine photocontact dermatitis, the magnitude of response depends on the strain of mice. In KP photocontact dermatitis, the responses of H-2^k strains, such as AKR, are higher than those of H-2^d strains, such as BALB/c [31]. On the other hands, H-2^d mice exhibit remarkably high responses in TCSA photocontact dermatitis, whereas H-2^k mice are low responders in this sensitivity [15]. Thus, the sensitivity seems to be controlled by MHC class II molecules. In this study, the augmented expression of MHC class II and CD86 was in accordance with the magnitude of KP or TCSA photocontact dermatitis in AKR and BALB/c mice. We further examined the difference in mRNA expression for IL-1 α and GM-CSF by epidermal cells between BALB/c and AKR mice phototreated with KP or TCSA. However, the cytokine expression of AKR mice was higher than that of BALB/c in not only KP but also TCSA phototreatment (data not shown). Thus, the difference in LC maturation and resultant sensitivity magnitude is not attributable to keratinocyte cytokine production.

Our study suggests two clinically important notions. First, nonsteroidal anti-inflammatory drugs are capable of functioning as photohaptens, thereby stimulating LCs to mature. In this process, keratinocytes are also stimulated to produce LC-stimulatory cytokines. Second, there are

differences among individuals in the susceptibility of LC responses to photohaptens upon UVA exposure. A number of reports have documented patients with severe photocontact dermatitis to KP [1,2,4]. In such cases, a predisposition to the photosensitivity might be closely associated with MHC haplotype.

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