表　ヒト凍結ヒト肝細胞によるヒト代謝経路の推定

薬　物	担当企業	*in vivo* 代謝及び代謝物	*in vivo* 生成比	ヒト肝細胞での代謝物	備　考
エノキサシン	三菱ウェルファーマ	ピペラジン環の酸化 (M2)、ピペラジン環の開裂 (M5) と N-脱アルキル化 (M1)、ピペラジン環の N-アセチル化 (M4) と酸化 (M3)	M2 > M5 > M4 = M1 > M3	M3、M5、M2、M4	
アザセトロン	三菱ウェルファーマ	N-脱メチル (M1)、N-酸化 (M2)	M2 > M1	M2 > M1	M2 は FMO による代謝産物
トログリタゾン	三共	Sul (M1)、Glu (M2)、Quinon (M3)	M1 > M3 > M2	M1、M3 > M2	
エストリオール	第一製薬	16-Glu (M1)、3-Glu (M2)、Glu-Sul (M3)、3-Sul (M4)	M1 > M2 > M3 > M4	M4、M1	
イミダプリル	第一製薬	イミダゾリン環の開裂 (M1)、ペプチド結合の加水分解 (M2、M3、M4)	M2 > M1 > M4 > M3	M1	加水分解は消化管で起こる
エトドラク	日本新薬	Glu (M1)、6-OH (M2)、7-OH (M3)、6-OH-Glu (M4)、7-OH-Glu (M5)	M1 > M5 > M3 > M2 > M4	M1 > M3、M5、M2	S体 > R体
リルマザホン	塩野義製薬	ペプチド結合の加水分解 (DG)、DG の縮合 (M1)、M1 の脱メチル (M2、M3)、M1-M3 の加水分解 (M4)	M4 > M2 > M1 > M3	M4 > M2 > M3	
ニトラゼパム	塩野義製薬	ニトロ基還元 (M1)、M1 のアセチル化 (M2)	M1 > M2	M1	嫌気的条件で M1 生成増加

Glu: glucuronide、Sul: sulfate、OH: hydoroxy

CYP2D6 の PM と EM の凍結ヒト肝細胞は IVT 社で調製されたものから選択し，遺伝多型を第一製薬（株）で確認したものを用いた．

DEX の N-脱メチル化代謝物 3-MEM と O-脱メチル化代謝物 DXO の生成量を測定した結果，両代謝物の比（3-MEM/DXO）は，EM-PM 間で大きな差が見られた．また，DXO の抱合体（glucuronide）も観察された．これらの結果は，定性的にヒトの臨床結果とよく一致していた．

同様の検討を，warfarin，bufuralol，mephenytoin，diclofenac，tramadol についても行った．その結果，いずれも変異の影響を確認できた．

5. まとめ

1) 手術摘出肝ブロックから肝細胞を調製できたが，肝臓の多くは病変が著しく，肝細胞の収量は少なかった．

2) ヒト肝細胞を用いることにより，①*in vivo* と同様の代謝物を半定量的に検出できる．第一相代謝と抱合反応の両方を検討できる．②肝特異的代謝を検討できる．③立体特異的な代謝を検討できた．④嫌気的条件での代謝を検討できた．⑤ロットにより大きなばらつきがあり，複数のロットでの検

討が必要である．

3) PM 凍結ヒト肝細胞を用いた代謝評価試験は，PM 患者のヒトの代謝パターンを把握するのに有用と考えられる．

4) データは示さなかったが，非凍結肝細胞を用いることにより，CYP1A，CYP3A の誘導能を検討できる．但し，ロット差が大きいことから，陽性対照物質との比較の上で評価する必要がある．

Photocontact Dermatitis: From Basic Photobiology to Clinical Relevance

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Review

Photocontact Dermatitis: From Basic Photobiology to Clinical Relevance

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Abstract   **Photocontact dermatitis is one of the major occupational and environmental skin diseases, which is adversely induced by chemicals. This dermatitis is evoked by skin application of photosensitive agents plus ultraviolet light irradiation. There are phototoxic and photoallergic mechanisms in this dermatosis. It is thought that the incidence of the latter is higher than that of the former. The photoallergic type is mediated by Langerhans cells, T cells, and various cytokines and chemokines, and thus occurs *via* a well-organized immunological mechanism. Photoconjugation of epidermal cells with a photohaptenic chemical is the initial step, and Langerhans cells serve as antigen-presenting cells. Causative photohaptens are bound to MHC class II molecules/self peptide on Langerhans cells upon exposure to UVA. The photomodified Langerhans cells sensitize and elicit antigen-specific T cells that mediate photoallergy.**
Key words : photoallergy, contact dermatitis, UVA, contact hypersensitivity, Langerhans cells

Introduction: Phototoxicity and photoallergenicity

In occupational skin disorders, contact dermatitis is the major skin disorder (1), and photocontact dermatitis is a specialized form of this skin disease (2). Eczematous eruptions that patients develop include erythema and papules/vesicles, and occasionally bullae, at the skin sites where a photocontactant is applied and solar light is irradiated. Histologically, the skin lesion is an eczematous tissue reaction characterized by epidermal spongiosis, exocytosis, and a dense mononuclear cell infiltrate in the dermis (3). Since the dermatitis is induced by a combination of application of a chemical to the skin and irradiation of the same site with ultraviolet (UV) light, it is sometimes difficult to diagnose this photosensitivity. The action

spectrum of this photosensitivity is mainly UVA.

Photocontact dermatitis is divided into two subtypes: phototoxic and photoallergic contact dermatitis. The phototoxicity is mediated by oxygen radicals, and the resultant cellular damage is caused by photoreaction of chemicals with lipid, DNA, and amino acids. Recently, the cellular phototoxicity against 3T3 fibroblasts has been used for evaluation of phototoxicity, and a study to validate this *in vitro* system is being performed in Japan as well as in some European countries. In contrast, the photoallergenicity is induced and elicited by immunological sequences involving many immunocompetent cells and molecules. Therefore, evaluation of photoallergenicity is more difficult than that of phototoxicity.

In photocontact dermatitis, the incidence of photoallergy is thought to be higher than that of phototoxicity. Thus, great attention must be paid to photoallergic contact dermatitis (4, 5). This review aims to highlight photocontact dermatitis focusing on its immunological and molecular mechanisms.

Table 1. Causative agents of photocontact dermatitis

Antimicrobial agents (mainly halogenated salicylanilides)
    tetrachlorosalicylanilide (TCSA)
    dibromosalicylanilide (DBS, dibromosalan)
    tribromosalicylanilide (TBS)
    bithionol (thiobisdichlorophenol)
    trichlorocarbanilide (TCC, triclocarban)
    trifluoromethyldichlorocarbanilide (TFC)
    hexachlorophene
    chloro-2-phenylphenol (Dowicide 32)
    fenticlor (thiobischlorophenol)
    multifingin (bromochlorosalicylanilide, BCSA)
    jadit (buclosamide, butylchlorosalicylamide)
    triclosan
    chlorhexidine
    dichlorophene
    sulfanilamide
Perfumes
    musk ambrette
    6-methylcoumarin
    sandalwood oil
Sunscreens
    para-amino-benzoic acid (PABA)
    octyl-dimethyl PABA (padimate O)
    amyl-dimethyl PABA (padimate A)
    glycerol PABA
    benzophenone (especially benzophenone-3=oxybenzone)
    butyl-methoxydibenzoylmethanes (Parsol 1789)
    digalloyl trioleate
    cinnamates (cinoxate)
Hair dye
    paraphenylenediamine (PPD)
Non-steroidal anti-inflammatory drugs
    Ketoprofen
    Suprofen
Phototoxic therapeutic chemicals
    Psoralen
    Coal tar

Causative chemicals

As shown in Table 1, various agents have been reported to evoke photocontact dermatitis. Historically, halogenated salicylanilide and related compounds, which were contained in soaps/detergents and used as topical antimicrobial agents, yielded a large number of patients with photocontact dermatitis (6-8). The elimination of these germicides from the market reduced the number of such patients. Perfumes, such as musk ambrette and 6-methylcoumarin, and sunscreen agents, especially benzophenone-3 (oxybenzone) (5), became causative thereafter. However, recent causative agents of photocontact dermatitis are topical non-steroidal, anti-inflammatory drugs, such as ketoprofen (9) and suprofen (10).

Almost all chemicals listed in Table 1 clinically evoke photoallergic dermatitis. However, when tested in their phototoxicity, they exhibit various degrees of phototoxicity. Therefore, it had been misestimated that phototoxicity is the mechanism of photocontact dermatitis in most cases. The agents that cause only phototoxicity with rare exceptions include psoralen and coal tar, which have thus for been used for therapy.

Photoantigen formation in photo-allergic type

Since photoallergic contact dermatitis is an immunological disorder, it is necessary for causative chemicals to become antigens or photoallergens upon exposure to UVA. As illustrated in Fig. 1, two hypotheses have been put forward to explain the formation of photoallergen (5). One is that the photosensitizer is a prohapten, which is converted to a complete hapten by UV irradiation, and the hapten can binds to protein. In another theory, the photosensitizer is a photohapten, which, in advance, binds noncovalently to the carrier protein, and upon UV irradiation, a covalent bond occurs *via* the formation of free radicals. In the case of photohaptens, therefore, UVA-preirradiated chemicals are incapable of binding to protein, and a non-covalent bond between photohapten and protein is required before irradiation of them with UVA.

This is in accordance with clinical photopatch test, in which a causative chemical is applied to the skin and UVA is irradiated to the same site. This method is for testing the photohaptenic property. When the prohaptenicity is examine, a UVA-preirradiated chemical should be ap-
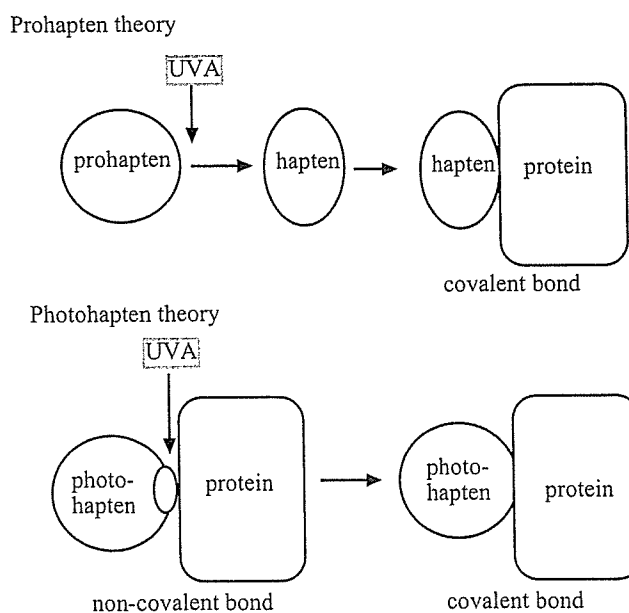

Fig. 1 Two theories for the formation of photoantigen

Table 2.   Prohapten and photohapten in photopatch test

	Photopatch test	Patch test of UV-preirradiated chemical
Prohapten	− (or +)	+
Photohapten	+	−

plied to the skin as patch test. Empirically, photopatch test has been performed to test the photoallergenicity of chemicals. Our studies have suggested that the vast majority of clinically photoallergic chemicals are photohaptens rather than prohaptens (11-13). Therefore, patients mostly exhibit positive photopatch test but negative patch test of the preirradiated chemical (Table 2).

Mouse model of photoallergic contact dermatitis

Mouse models of photoallergic contact dermatitis were established by several groups in the early 1980's (14, 15), and enabled researchers to elucidate mechanisms of the sensitivity, because of its technical convenience and availability of accumulated immunologic information on this species. In these models, 3,3',4',5-tetrachlorosalicylanilide (TCSA), a representative halogenated salicylanilide, has been used typically as photohapten. Mice are sensitized by 2 daily abdominal paintings with 1% of TCSA plus UVA irradiation and challenged 5-days later on the earlobes with TCSA plus UVA. Ear swelling responses are measured 24 h after challenge. In addition to TCSA, the photoallergenic potential of other halogenated salicylanilides, such as tribromosalicylanilide and bithionol, is also detected by this mode of sensitization (16).

Murine photoallergic contact dermatitis to TCSA is genetically controlled and determined mainly by the major histocompatibility complex (MHC) (17, 18). Mice with H-2[b,d] alleles are high responders, whereas the H-2[k] haplotype is closely associated with low responders (18). Such a clear-cut association of the H-2 haplotype with the degree of response has not

been found in ordinary contact dermatitis to haptens. We have recently found that in photoallergic contact dermatitis to ketoprofen, a non-steroidal anti-inflammatory drug, mice with H-2[k] are high responders, whereas those with H-2[b,d] are low responders. Therefore, high responder H-2 haplotypes differ among photosensitivities to each photohaptenic chemical.

Immunological mechanism of photoallergic contact dermatitis

UVA is the action spectrum of this photoderivatization, as protein and cells are photocoupled with a photohapten by irradiation with UVA but not UVB. The main sequential events in photoallergic contact dermatitis are virtually the same as those of ordinary contact dermatitis except for the requirement of UV irradiation in sensitization and challenge (Fig. 2). Photoconjugation of epidermal cells with TCSA is the initial step in the photoallergy. Langerhans cells (LC), which are professional antigen-presenting cells in the epidermis, play an important role and T cells sensitized by photohapten-bearing LC induce this photosensitivity (2). Migration of TCSA-bearing LC to draining lymph nodes in the sensitization phase (19) and involvement of mast cells in the challenge phase (20) are requirements.

Murine photoallergic contact dermatitis to TCSA involves both positive and negative immunologic pathways that are restricted by I-A and I-E molecules on antigen-presenting cells (18, 21). The suppressive pathway is mediated by IL-10-producing Th2 cells (21), which had been known as suppressor T cells and may correspond to recently called regulatory T cells. Sensitization with TCSA plus UVA is prone to induce Th2 cells compared to ordinary haptens
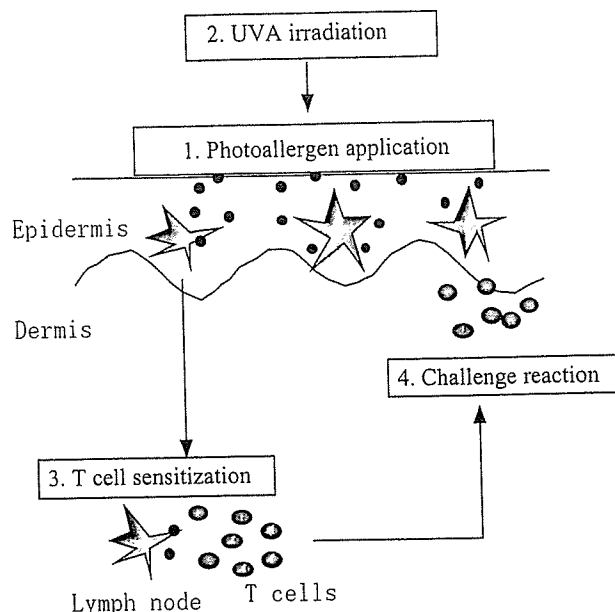
Fig. 2   Immunological mechanism of photoallergic contact dermatitis

(22), suggesting that the suppressive immuno-logic pathway is clearly detectable in this sensi-tivity. Antigen-specific, afferent limb-acting Th2 or regulatory T cells are responsible for the low responsiveness of $H-2^k$ mice. The low re-sponsiveness of photoallergic contact dermatitis in the $H-2^k$ strain is due to the preferential acti-vation of Th2 or regulatory T cells via $I-E^k$ molecules (18).

Molecular mechanism of photoantigen presentation

In photoallergic contact dermatitis and drug photoallergy, causative photohaptens are bound to MHC class II molecules/self peptide on LC upon exposure to UVA (23). The photo-modified LC sensitize and elicit antigen-specific T cells that mediate photoallergy (24). In our murine model of fluoroquinolone photoallergy, quinolone diffuses to the epidermis. Upon UVA exposure, LC are photomodified with a given quinolone in their MHC class II-associated pep-tides, thereby sensitizing and eliciting TCR V β 13-bearing T cells (25), which lead to photo-allergic skin reactions. It is possible that pro-tein is covalently bound to a photodegradated

site of photohapten to form an allergic photohapten-protein complex (25). Lysine is a preferential amino acid to afford the binding site to a photohapten (23). Primed $CD4^+$ T cells proliferated in vitro in response to LC loaded with class II $(I-A^d)$-binding, lysine-containing peptides when photomodified with a photo-hapten (23). Epicutaneous application of the FQ-photoconjugated peptide via barrier-disrupted skin was able to sensitize mice for subsequent elicitation of photoallergy evoked with systemic photohapten and UVA.

Topical application of TCSA and UVA irra-diation not only produce the formation of photoantigen but also promote the antigen-presenting ability of LC. The combination of TCSA painting and UVA exposure markedly elevates the expression of MHC class II and CD86 and slightly that of CD80 and CD54 on the surface of LC (26). There exist sub-populations of LC that express MHC class II and CD86 at high levels. Since neither TCSA painting nor UVA exposure alone enhances the expression, both treatments are essential for enhancement. MHC class II and CD86 mole-cules are mandatory for the antigen-presenting function of LC. Therefore, as ordinary haptens (27), photohaptens are capable of inducing immunocompetent molecules on antigen-

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presenting cells when irradiated with UVA.

Future study: Screening of photo-allergenicity using *in vitro* system

The phototoxicity of given substances has been evaluated in many *in vitro* systems, and the standard system is now being established by using cellular phototoxicity. However, the photoallergenicity is not easily evaluated in either *in vivo* or *in vitro* system. In particular, it is very difficult for an *in vitro* system to predict photoallergenicity. Nonetheless, two possibilities may be proposed for the prediction. One is to test photoconjugation of given substances with protein, because this binding is the initial step of photoallergy in the skin and yields photoantigens. Liquid chromatography or mass spectrometry is a choice to examine the conjugation. In the other system, some cell lines with antigen-presenting ability, such as THP-1, can be used for *in vitro* treatment with a photoallergic substance plus UVA. When the phototreated cells express higher levels of MHC class II and costimulatory molecules, such as CD86, CD80 or CD40, the substance will have an ability to phosensitize and photoelicit T cells. These systems may shed light for future studies.

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LETTER TO THE EDITOR

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The role of low-density lipoprotein receptors in sensitivity to killing by Photofrin-mediated photodynamic therapy in cultured human tumor cell lines

Photodynamic therapy (PDT) is a newly developed modality for the treatment of patients with cancer [1]. Recently a partially purified fraction of the hematoporphyrin derivatives, Photofrin[®], has frequently been used as a photosensitizer in PDT. There are several rationales for which Photofrin-PDT exerts its therapeutic effectiveness: (1) Photofrin accumulates selectively into cancer cells through low-density lipoprotein receptors (LDL-R); (2) reactive oxygen species (ROS) generated after the photoexcitation of Photofrin by laser irradiation destroy tumor cells; (3) vascular tissues necessary for tumor growth can be injured by Photofrin-PDT, resulting in the occlusion of vessels [2—6]. However, the precise mechanisms of Photofrin-PDT-induced tumor-cell killing have not yet been fully elucidated. The important clinical aspect is to clarify whether Photofrin-PDT sensitivity differs among various tumors and to predict which types of tumors are more effectively treated with Photofrin-PDT. Furthermore, the significance of LDL-R at the surface of tumor cells for the incorporation of Photofrin and the resultant effectiveness of Photofrin-PDT still remains controversial [2,7,8]. To better understand Photofrin-PDT in the above respects, we examined the effectiveness of in vitro Photofrin-PDT in cultured human tumor cell lines and analyzed the sensitivity of Photofrin-PDT in relation to their expression of LDL-R and uptake of Photofrin.

Human tumor cell lines and a normal fibroblast cell line used in the study are listed in Table 1. The cells were maintained in Dulbecco's modified minimum essential medium (DMEM) (Sigma Chemical CC, Tokyo, Japan) with 10% fetal bovine serum (Filtron, Australia) at 37 °C in a 5% CO_2 atmosphere.

Immunohistochemically, the expression of LDL-R in squamous cell carcinoma (SCC) cells (HSC-2) and adenocarcinoma cells (MKN45) was high, while melanoma cell lines (G361, Mewo and C32TG) and normal cells (N-1) bore the receptor at very low or even undetectable levels by use of polyclonal antibody to human LDL-R (working dilution; I:10, Progen, Heiderberg, Germany). Using the fluorescence microscopy [9], the uptake of Photofrin (Wyeth-Lederle, Japan, 50 μg/ml at 37 °C for 1 h in DMEM) into the cells was higher in SCC cells (HSC-2) and relatively higher in adenocarcinoma cells (MKN45) than that in melanoma cells (G361, C32TG). The fluorescence of Photofrin was barely detectable in normal cells (N-1). The intensity of fluorescence in MKN45 was detectable but less intensive than that in HSC-2. When HSC-2 and G361 were treated in the presence of LDL (0.5 mg protein/ml in DMEM, Sigma—Aldrich, St. Louis, MO, USA) with Photofrin, the uptake of Photofrin into SCC cells (HSC-2) was much decreased with the LDL treatment compared to that without LDL treatment. However, HDL (1 mg protein/ml in DMEM, Sigma—Aldrich, St. Louis, MO, USA) did not affect the uptake of Photofrin in SCC cells (HSC-2).

Cells were treated with Photofrin (50 μg/ml at 37 °C for 1 h in DMEM), washed by phosphate-buffered saline and irradiated with an optical parametric oscillator (OPO) laser (Hamamatsu Photonics K.K., Hamamatsu, Japan) emitting 7.3 mW/cm² of the energy fluence rate at 630 nm. At this irradiance, the hyperthermic effect of Photofrin-PDT was negligible. Five days after the treatment, cell survival was determined as described previously [10]. Survival was expressed as the ratio of cell counts in the treated dishes to that in the control (Photofrin treatment without laser irradiation) dishes. Survival affected by laser irradiation without Photofrin treatment was also measured. Our preliminary study showed that 50 μg/ml or less of Photofrin treatment for 1 h without subsequent laser irradiation had no cytotoxic effect on HSC-2. As shown in the figure, in vitro Photofrin-PDT killed cultured tumor cells and the sensitivity to Photofrin-PDT varied widely among the cultured human cell lines. The post-Photofrin-PDT survivals of human SCC (HSC-2 and HSC-3) and

Table 1 Characteristics of cell lines used in the study

Cell strain	Type of tumor (histopathology and origin)	Sources#	LDL-R*1	Uptake of Photofrin*2		
				No treatment	+LDL	+HDL
HSC-2	Squamous cell carcinoma (lip)	JCRB (0622)	++	++	±	++
HSC-3	Squamous cell carcinoma (tongue)	JCRB (0623)	n.d.	n.d.	n.d.	n.d.
Mewo	Melanoma (lymph node)	JCRB (0066)	−	n.d.	n.d.	n.d.
G361	Melanoma (skin)	JCRB (9074)	−	±	−	±
C32TG	Amelanotic melanoma (skin)	JCRB (0227)	−	±	−	±
MKN45	Adenocarcinoma (stomach)	JCRB (0254)	+	n.d.	n.d.	n.d.
N-1	Normal fibroblast (skin)	Established in our laboratory	−	−	−	−

(#) JCRB, Japanese cancer reseach resources bank. The number in parentheses represents the catalog number of the cells from JCRB; *1 and *2 level of immunohistochemical expression of low-density lipoprotein receptor (LDL-R) and intensity of fluorescence from Photofrin, respectively; (++) strongly positive in all of the cells; (+) weakly positive in all of the cells; (±) weakly positive in some (less than 50%) of the cells; (−) negative in all of the cells; n.d., not determined.

adenocarcinoma (MKN45) cell lines were significantly lower at 2.5 J/cm^2 compared to those of the melanoma lines (Mewo, G361, and C32TG). Both Mewo and G361 were highly melanin-producing cells, whereas C32TG cells produced little amount of melanin. Since survivals of these three melanoma lines were comparable, the amount of melanin did not affect the susceptibility to the present protocol of Photofrin-PDT. In the normal fibroblast cell line, N-1, Photofrin-PDT exerted a modest lytic effect and their sensitivity was comparable to that of Mewo. On the contrary, survivals were more than 95% in all of the cell lines tested after laser irradiation without Photofrin pre-treatment. The representative data of HSC-2, Mewo and N-1 are depicted in Fig. 1.

In this study, we demonstrated that SCC and adenocarcinoma cells were more susceptible than melanoma lines. It has been postulated that melanin is one of the factors that account for the resistance of melanoma cells to the Photofrin-PDT, because this molecules not only protects the intracellular organelles from the visible light but also scavenges Photofrin-PDT-induced ROS, dampering cellular damages by PDT. However, the present study showed no difference in cell survival after Photofrin-PDT between melanotic and amelanotic melanoma cells. This observation suggested that the amount of melanin in the cells did not affect the sensitivity to killing by PDT. On the other hand, both melanotic and nonmelanotic melanoma cells were found to minimally express LDL-R. The expression of LDL-R in SCC and adenocarcinoma cells was higher than melanoma cells. By fluorescence microscopy, the uptake of Photofrin was high in the cells with strong expression of LDL-R, while low LDL-R expression was correlated with minimal incorporation of Photofrin. Furthermore, Photofrin fluorescence was barely observed in the presence of LDL, not HDL, implying that exogenous LDL occupied LDL-R and did not enable Photofrin to access them. These data confirm that the sensitivity to Photofrin-PDT among tumors depends on the levels of Photofrin incorporated through LDL-R. Our study demonstrated that Photofrin was easily introduced into cells with high LDL-R compared to cells with the low LDL-R expression, suggesting that Photofrin-PDT sensitivity of cells stems partly from the ability of incorporation of Photofrin through LDL-R. Our data also suggest that the efficacy of Photofrin-PDT can be predicted by examining the LDL-R expression in biopsy specimens of tumors.
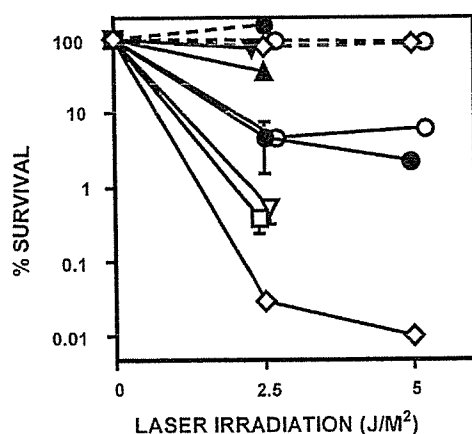

Fig. 1 Post-Photofrin-PDT survivals in human tumor cell lines. HSC-2 (◇), HSC-3 (□) MKN45 (▽), Mewo (●), G361 (▼) and C32TG (▲) cells and N-1 (○) as a control were treated with Photofrin and subsequently exposed to laser and cell survivals were examined (solid lines). Survivals of HSC-2 (◇), Mewo (●) and N-1 (○) cells without Photofrin treatment were also depicted with dashed lines. Each point represents an average of four dishes. Vertical bars represent the mean ± S.D. In most of the points, the S.D.s are smaller than the symbols.

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Determination of action spectrum for sparfloxacin-photosensitized single-strand breaks in plasmid pBR322 DNA

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Background: Various drugs have been reported to induce photosensitivity as a side effect. Sparfloxacin (SPFX) is well known to trigger dermatological phototoxicity upon solar radiation exposure.
Purpose: To prevent SPFX-induced phototoxicity, we determined the wavelength range responsible for SPFX phototoxicity.
Methods: The action spectrum for SPFX photosensitization was assessed by the formation of single-strand breaks in plasmid pBR322 DNA.
Results: The wavelengths of light leading to the formation of single-strand breaks were in the ultraviolet A (UVA) and visible ranges. In comparison with the absorption spectrum, we found that SPFX absorption primarily contributed to the action spectrum of single-strand break formation, but it even expanded to the visible range (between 320 and 480 nm) beyond the absorption wavelengths.
Conclusion: The findings suggest that protection of skin from short wavelengths of visible light beyond the absorption wavelengths as well as UVA light is of primary importance in prevention against induction of SPFX phototoxicity.

Key words: action spectrum; photoprotection; photosensitization; plasmid DNA; single-strand breaks; sparfloxacin.

A wide variety of new synthetic drugs have been developed to apply to clinical treatments. However, because of the frequent use of these drugs in aged populations, drug-induced photosensitivity has been a serious problem as a side effect. Fluoroquinolone antibacterial agents are known to be typical photosensitive drugs (1, 2). Both phototoxicity and photoallergy play a role in the pathogenesis of fluoroquinolone photosensitivity (2, 3), depending on their chemical structures (2). The phototoxic property has been reported to be determined at least partly by a substituent at the 8-position of the structural backbone (2, 4). In an animal model, reactive oxygen species are involved in the phototoxicity (5). Other accumulated literatures on fluoroquinolne phototoxicity in clinical and laboratory studies have been reviewed (6). Among fluoroquinolones, sparfloxacin (SPFX) has been reported to cause a high incidence of photosensitivity, which has been shown to be phototoxic but not photoallergic reactions (1, 2, 7–9).

The fundamental way to prevent this side effect is photoprotection of skin from its action spectrum. While it is not easy to determine the action spectrum of a given phototoxic drug by using the cutaneous response, its *in vitro* phototoxicity can be assessed easily and quantitatively by detecting the photosensitized formation of plasmid DNA strand breaks (7, 10–14). *In vitro* photochemical studies on SPFX phototoxicity have mainly focused on plasmid DNA strand breakage (7, 10, 14). The induction of DNA strand breakage by SPFX has been shown to relate to singlet oxygen molecules generated by SPFX photoexcitation (10). A possible involvement of DNA damage in SPFX phototoxicity is suggested by the accumulation of SPFX in the nuclei of human buccal mucosa cells (15).

In this study, we used the *in vitro* DNA strand-breaking activity as a useful indicator of SPFX phototoxicity. The results demonstrate that the action spectrum for SPFX photosensitized formation of single-strand breaks in plasmid pBR322 DNA

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encompasses from the ultraviolet A (UVA) to the short wavelength range of visible light.

Materials and methods

Materials

Plasmid pBR322 DNA was a product of MBI Fermentas (Vilnius, Lithuania). SPFX [CAS: 110871-86-8] was kindly provided by Dainippon Pharmaceutical Co. Ltd. (Osaka, Japan). The structural formula is shown in Fig. 1. Agarose ME was purchased from Iwai Chemicals Company Ltd. (Tokyo, Japan). Ethidium bromide was from Nippon Gene Co., Ltd. (Tokyo, Japan), and Tris was from Sigma Chemical Co. (St. Louis, MO, USA). Tris-HCl, EDTA·2Na, bromophenol blue, glycerol, and 5 × TBE buffer (89 mM Tris, 89 mM boric acid, and 2 mM EDTA; pH 8.3) were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Absorption spectrum of SPFX

SPFX was dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA·2Na, pH 8.0) containing 0.4% N,N-dimethylformamide (DMF). DMF was added to solubilize SPFX in TE buffer. The concentration of SPFX was 50 µM. The absorption spectrum of SPFX was measured with a spectrophotometer (Model MPS-2000, Shimadzu Co., Kyoto, Japan) at 25 ± 0.5 °C.

UVA irradiation with BLB lamps

The sample solution containing plasmid pBR322 DNA and SPFX prepared in TE buffer was irradiated with BLB lamps to measure the UVA fluence dependence of single-strand breaks. The final concentrations of the DNA and SPFX were 5.55 µg/ml and 3.5 µM, respectively. A glass capillary (Drummond Scientific Co., Broomall, PA, USA) was filled with the solution (10 µl). Note that the glass does not absorb in UVA light. The temperature of the solution was maintained at 25 °C throughout the course of UVA irradiation. Irradiation of the solution was performed with two BLB lamp tubes arranged in parallel (Nominal lamp power: 32 W, specially manufactured by Nippo Electric Co, Ltd., Kanagawa, Japan) under air-saturated conditions. The lamp had a 300–420 nm emission range with the maximum at 350 nm. The UVA irradiance was measured with a UV radiometer (Model UVR-36, Topcon Co., Tokyo, Japan) to be 30 or 50 W/m^2.

Monochromatic light irradiation

To measure the action spectrum for formation of single-strand breaks, the sample solution (10 µl) in the same type of capillary as described above was
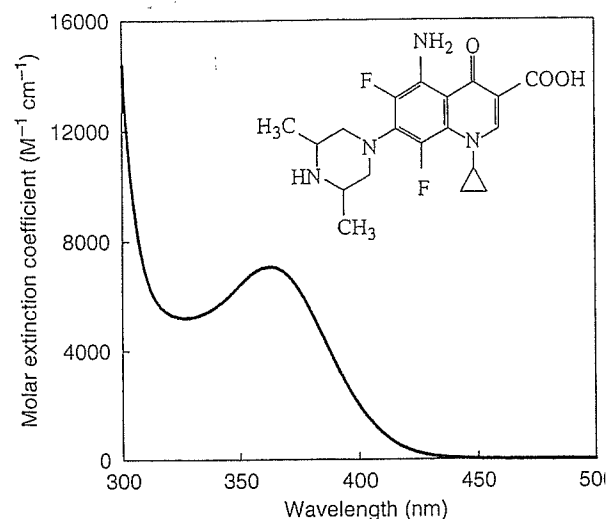

Fig. 1. Absorption spectrum of sparfloxacin (SPF) (concentration: 50 µM) in TE buffer, containing 0.4 dimethylformamide, and the structural formula SPFX.

irradiated with monochromatic light at different w velengths from 320 to 480 nm. The solution contain the plasmid DNA (5.55 µg/ml) and SPFX (10 µM dissolved in TE buffer. Monochromatic light irradi tion of the sample was performed by means of 'spectroirradiator' (Model MLS-121, ORC MAN FACTURING CO, LTD., Tokyo, Japan). The app ratus used consists of a 1 kW xenon short arc lan optically coupled to a single grating monochromat The three capillaries were fixed with a holder set on t exit of the monochromator in parallel, and uniform irradiated with monochromatic light at room tei perature under air-saturated conditions. The appai tus was set to irradiate monochromatic light at 3 326, 340, 360, 375, 400, 440 and 480 nm, respective The spectral resolution was 4 nm. Several kinds of ct off filters were used to reduce shorter wavelengths scattered light for the longer wavelength monochi matic light. The values of irradiance were measur with an IL1745 UV Curing Radiometer System (Int national light Inc, Newburyport, MA, USA), as 17 m^2 (320 nm), 21 W/m^2 (326 nm), 32 W/m^2 (340 n 48 W/m^2 (360 nm), 60 W/m^2 (375 nm), 79 W/ (400 nm), 44 W/m^2 (440 nm) and 48 W/m^2 (480 nm)

Agarose gel electrophoresis

Separation of the different forms of plasmid pBR DNA, i.e., the closed circular form (native conforn tion), the open circular form (resulting from sing strand breaks) and the linear form (resulting fr double-strand breaks), was performed by horizon agarose gel electrophoresis (1.1% agarose slab gels TBE buffer. The irradiated solution (2 µl) was mi

with 1 μl of loading buffer (0.25% bromophenol blue, 30% glycerol in TBE buffer). The mixed solution was applied to wells in the slab and electrophoresed with a Mupid-3 system (Cosmo Bio Co., Ltd., Tokyo, Japan) for 40 min at 100 V. After electrophoresis, the gels were stained with ethidium bromide (0.5 μg/ml) for 30 min and rinsed with 0.5 × TBE buffer for 5 min. The DNA forms that were separated by electrophoresis were visualized by fluorescence of ethidium bromide, which was intercalated with the DNA forms, on a transilluminator (Model TFP-10 M, Vilber Lourmat, Marne-La-Vallée Cedex, France). The fluorescent patterns were photographed on a Polaroid-type 667 positive film through a red filter. The resulting photographs were scanned into a computer and processed using Scion Image program (Scion Co., Frederick, MD, USA). The ratios of the relative amounts of the open circular form to the closed circular form were calculated from the peak areas of the densitometric trace of the fluorescence image. In addition, as the fluorescence intensity of ethidium bromide bound to the closed circular form was less than that bound to the open circular form by a factor of 1.42 (16), amounts of the closed circular form were corrected by this factor.

Results

Absorption spectrum of SPFX

The absorption spectrum of SPFX in TE buffer containing 0.4% DMF was measured as shown in Fig. 1. SPFX had an absorption band in the range of 320–450 nm, with a peak at 362 nm. The molar extinction coefficient at the absorption maximum was $7100 \, M^{-1} \, cm^{-1}$.

DNA strand-breaking activity of UVA irradiation with SPFX

The DNA strand-breaking activity of photosensitized SPFX was identified by 1.1% agarose gel electrophoretic separation of the DNA forms after irradiation with the BLB lamps for 20 min, 40 or 60 min. When the plasmid DNA was irradiated in the presence of SPFX, single-strand breaks in the DNA were produced, converting the closed circular form into the open circular form (Fig. 2). The fluorescence intensities of ethidium bromide in the electrophoretic bands depend on the concentrations of the DNA forms. The photocleavage efficiency was increased with increasing UVA-irradiated fluence (Fig. 3). The amount of the closed circular form decreased exponentially with increasing fluence, whereas the amount of the open circular form increased conversely. To analyze these data using the hit theory, the values of
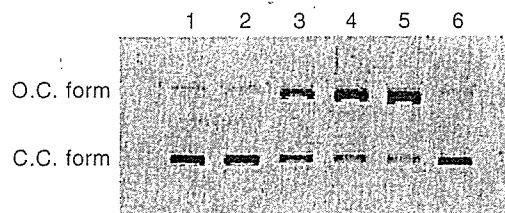

Fig. 2. Electrophoretic patterns on 1.1% agarose gel showing single-strand breaks in plasmid pBR322 DNA by sparfloxacin (SPFX) photosensitization. Concentration of SPFX, 3.5 μM; light source, BLB lamps; irradiance, 50 W/m². C.C., closed circular; O.C., open circular; lane 1, dark control (−SPFX); lane 2, dark control (+SPFX); lane 3, fluence 60 kJ/m² (+SPFX); lane 4, fluence 120 kJ/m² (+SPFX); lane 5, fluence 180 kJ/m² (+SPFX); lane 6, fluence 180 kJ/m² (−SPFX).
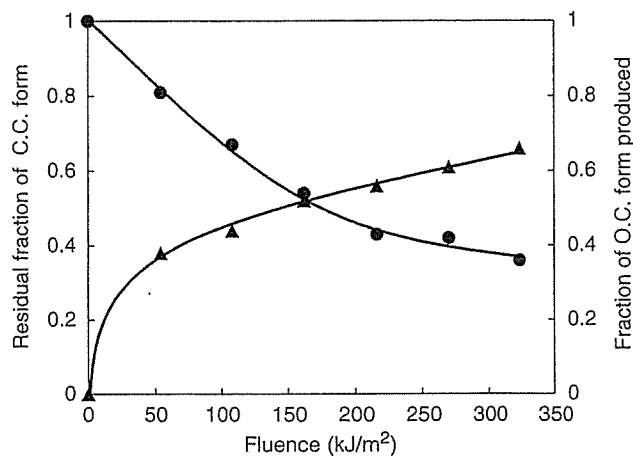

Fig. 3. Change of residual fraction of CC form and fraction of OC form produced with increasing fluence. Concentration of sparfloxacin, 3.5 μM; light source: BLB lamps; irradiance, 30 W/m²; C.C., closed circular (●); O.C., open circular (▲).

−ln (fraction of closed circular form) were plotted as a function of UVA-irradiated fluence. As shown in Fig. 4, the change in the residual fraction of the closed circular form fitted to the single-hit kinetics in the range of 0–162 kJ/m². No significant electrophoretic band corresponding to the linear form resulting from double-strand breaks was observed in the irradiated fluence range examined. As a dark control experiment, an unirradiated mixture of the DNA and SPFX was also analyzed electrophoretically. The closed circular form in the mixture migrated at the same rate as the DNA in the absence of SPFX, showing that no electrophoretically detectable modification of the DNA was induced by addition of SPFX in the dark (dark control). Irradiation of the DNA in the absence of SPFX (irradiated control) did not alter the electrophoretic
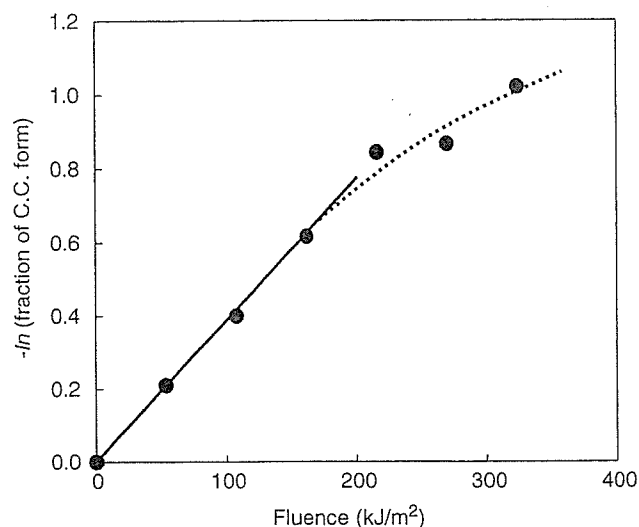
Fig. 4. Fluence dependence of the photosensitized single-strand breaks. Concentration of sparfloxacin: 3.5 μM, Light source, BLB lamps; Irradiance, 30 W/m^2; C.C., closed circular.

pattern under the present experimental conditions. These findings indicate that SPFX can behave as a UVA photosensitizer for induction of the photosensitized formation of strand breakage in the DNA.

Action spectrum for the SPFX-photosensitized formation of single-strand breaks

The action spectrum for the SPFX-photosensitized formation of single-strand breaks was determined for the 320–480 nm range. As mentioned above, the lesion in the range of a low fluence was recognized to follow the single-hit kinetics with respect to the fluence. This relation was reconfirmed in cases of monochromatic irradiation (Fig. 5). The lines illustrated in the figure indicate best linear regressions constrained through the origin. The result enabled us to compute the action cross-section of the photocleavage caused by irradiations at different wavelengths. The data were obtained after the correction with respect to the irradiated control experiment. Consequently, the action spectrum for the SPFX-photosensitized formation of single-strand breaks was determined as shown in Fig. 6. The maximum response was observed at around 370 nm. As illustrated, the maximum response shifted slightly to longer wavelengths when compared with the absorption spectrum. In addition, extension of the action spectrum to the visible region beyond the absorption wavelengths was apparent.

Discussion

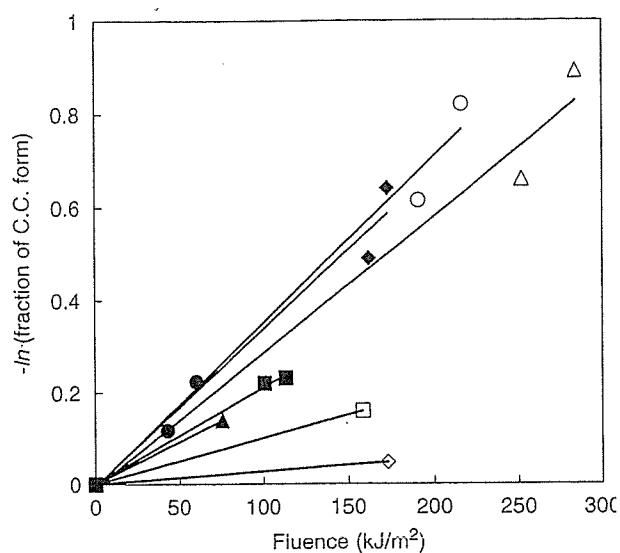We presented the action spectrum for the *in vitro* formation of single-strand breaks in plasmid pBR322

Fig. 5. Fluence dependence of the photosensitized single-strand breaks by irradiation with monochromatic lights. Concentration of sparfloxacin 10 μM; Light source, the xenon short arc lamp C.C., closed circular; ●, 320 nm; ▲, 326 nm; ■ 340 nm; ◆, 360 nm; ○, 375 nm; △, 400 nm; □ 440 nm; ◇, 480 nm.
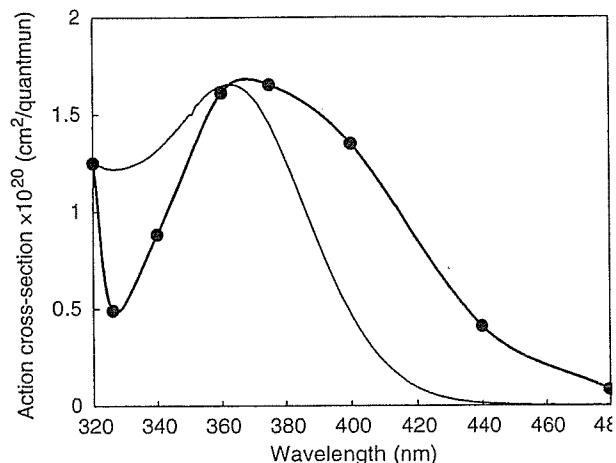

Fig. 6. Action spectrum for the photosensitized singl strand breaks. The spectrum was superimposed on tl relative absorption spectrum of sparfloxacin. (—●— action spectrum; (———), absorption spectrum.

DNA by photosensitization with SPFX. The acti spectrum was largely reflective of the absorption spe trum. The major wavelengths were in the UVA ran but visible light beyond the absorption wavelengt was also found to be involved in the formation single-strand breaks in the plasmid DNA. The prese result has a clinical meaning in selection of the wav length range of light to be blocked for prevention SPFX-induced phototoxicity.

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A key point of the action spectrum obtained in the present study is the extension to visible range beyond the absorption wavelengths. It has been shown that at least three photoproducts are generated by irradiation of SPFX with UV light from a high-pressure Hg discharge lamp (17). Although their absorption characteristics are not known, it is likely that the photoproducts are involved in the extension of the action spectrum, given that they absorb light at longer wavelengths than SPFX and form single-strand breaks. A slight increase in the absorption of UVA-irradiated solution of SPFX alone or that of SPFX with calf thymus DNA in the visible range beyond the absorption wavelengths may be a line of evidence supporting the generation of the photoproducts (data not shown). Further work has to be carried out to resolve the cause of the extension. Although photoaugmentation with UVA and UVB has been shown to trigger SPFX phototoxicity (7), it is suggested that visible light has also been implied as an inducer of SPFX phototoxicity on the basis of its action spectrum. Occurrence of strand breakage observed by irradiation with a commonly used fluorescent lamp with a broad visible emission spectrum supports the present findings (7).

The induction mechanisms of SPFX-photosensitized DNA damage have been studied in some papers. For example, plasmid DNA strand breakage caused by photosensitization with SPFX was demonstrated to be mainly a TypeII photosensitized reaction (10), while singlet oxygen molecules generally do not induce direct strand breakage both within isolated and cellular DNA (18). Involvement of hydroxyl radicals in SPFX-photosensitized oxidative damage to DNA in retinal pigment epithelial cells was also proposed (19). Photoexcited fluoroqinolones other than SPFX have been shown to react with DNA directly in addition to the generation of active oxygen species. For example, the production of cyclobutane pyrimidine dimers through the triplet energy transfer mechanism upon UVA excitation (20), and a possible involvement of carbene intermediates in DNA strand breakage (13, 21), were shown. Thus, it is most likely that photosensitized DNA damage in the presence of SPFX occurs by way of a variety of mechanisms.

We have assessed quantitatively the preventive abilities of conventional clothes and commercial sunscreens against SPFX phototoxicity, referring to the photosensitized strand breakage in the plasmid DNA in vitro (22, 23). The present study supported the validity of adopting SPFX-photosensitized strand breakage as an indicator of UVA protection.

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Establishment of murine model of allergic photocontact dermatitis to ketoprofen and characterization of pathogenic T cells

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Summary

Background: Ketoprofen is well known to evoke the allergic type of photocontact dermatitis when it is applied to the skin and irradiated with ultraviolet A (UVA) light. Objective: We aimed to establish a murine model of this photosensitivity and to characterize pathogenic T cells concerned with the sensitivity. Methods: Various strains of mice were sensitized on two consecutive days by application of ketoprofen to the shaved abdomen and irradiation of the skin with UVA. Five days later, they were elicited with ketoprofen plus UVA on the earlobes. Immune lymph node cells and epidermal cells from the challenged sites were analyzed by RT-PCR. Results: Mice were successfully sensitized and challenged with 4% and 2% ketoprofen, respective, plus UVA at20 J/cm^2. The responses in H-2^k mice were higher than those in the other strains examined. Immune lymph node CD4$^+$ or CD8$^+$ cells from ketoprofen-photosensitized H-2^k mice were transferred i.v. to naïve syngeneic recipients. Mice receiving CD4$^+$ but not CD8$^+$ cells exhibited ketoprofen photosensitivity, but transference of both CD4$^+$ and CD8$^+$ cell populations was more effective. Lymph node cells from photosensitized mice expressed high levels of mRNA for Th2 cytokine (IL-4) and Th2 chemokine receptor (CCR4) as well as Th1 cytokine (IFN-γ) and Th1 chemokine receptor (CXCR3), as assessed by RT-PCR. In addition, epidermal cells from challenged earlobes expressed increased levels of both Th1 (TARC) and Th2 (Mig) chemokines. Conclusion: It is considered that not only Th1 but also Th2 cells participate in the pathogenesis of murine photocontact dermatitis to ketoprofen.

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