表2　判定基準

本邦基準[6]	反応	ICDRG基準	反応
−	反応なし	−	反応なし
±	軽度の紅斑	＋？	紅斑のみ
＋	紅斑	＋	紅斑＋浸潤，丘疹
＋＋	紅斑＋浮腫，丘疹	＋＋	紅斑＋浸潤＋丘疹＋小水疱
＋＋＋	紅斑＋浮腫＋丘疹＋小水疱	＋＋＋	大水疱
＋＋＋＋	大水疱	IR	刺激反応
		NT	施行せず

＋＋以上を陽性反応とする　　＋以上を陽性反応とする

分子量の大きい抗生物質やステロイド外用薬などの抗炎症作用のある物質は陽性反応が4日，もしくはそれ以上遅れて誘発されること，また高齢者は若年者に比べ陽性反応が遅れて出現する傾向があること，などが挙げられる。

6．判定基準（表2）

現在，アレルギー反応を判定・評価するためのパッチテスト判定基準には本邦基準[16]とICDRG基準[14]がある。本邦基準では刺激反応とアレルギー反応の区別の記載がないため，アレルギー反応の判定基準としてはICDRG基準が適している。アレルギー反応はパッチ除去後も反応が長く持続し，刺激反応は時間とともに反応が弱まっていく傾向がある。

7．パッチテストの解釈

判定結果と臨床症状の関連性を確認する。陽性反応が得られた場合は，①接触または使用歴を確認し，現在の皮膚炎の原因か増悪因子かを明らかにする。②今回接触した物質でなければ過去の皮膚炎の既往を十分に問診し，以前の皮膚炎の原因か，増悪因子かを明らかにする。③さらに，これまでの皮膚炎とは関係のない交叉反応である可能性を考慮する，などを検討する。一方，パッチテストが陰性であっても即座にアレルギー反応ではないと判断せず，アレルゲンを正しい濃度で適切に貼布したかなどを検証する。

1）多数の陽性反応が得られた場合

非常に強い陽性反応は，その反応の近傍にも陽性反応を出現させることがある。これをexcited skin syndromeもしくはangry back syndromeという。このような場合は，非特異的反応を排除するためにより少ない抗原数で再検査を行う[15]。

2）結果と臨床症状が一致しない場合

適切な濃度・基材で検査を行ったか検証し，可能な限り再検査を行う。また，パッチテストを実施するなかで偽陽性，偽陰性反応が生ずる場合があるため考慮する。

治療

接触皮膚炎の治療指針のアルゴリズムを図4にまとめた。接触皮膚炎の治療では原因となるアレルゲン，接触刺激因子を確定し除去することである。病歴の聴取により，推定された最も可能性の高い原因物質を除去することが重要である。炎症に対する治療法は，全身性と局所性の接触皮膚炎で異なる。全身性接触皮膚炎，接触皮膚炎症候群ではステロイド外用（推奨度A）とともに抗ヒスタミン薬（推奨度B），ステロイド内服薬（プレドニゾロン20 mg 日）（推奨度A）などのも第一選択のひとつとなりうるが[注]，限局性接触皮膚炎ではステロイド外用，保湿剤の外用（推奨度B）とともに日常品，化粧品では代替品の推奨などが必要である。限局性のときは，ステロイド内服薬は重症のときに限られ[注]，抗ヒスタミン薬は補助的療法となる。原因除去，排除とステロイド外用薬を主体とした治療法で2週間以内に軽快しないときは，まだ原因物質が不明で除去されていない可能性，もしくは原因物質が生活環境，職場にある可能性を考えパッチテストなどの原因特定の検査が必要となる。検査により原因が特定できたときには原因を含む物質，交叉性のある物質を排除・回避する。特定できないときには治療しているステロイド外用薬による接触皮膚炎の可能性も疑う必要がある。石鹸，洗剤による手湿疹などでは原因に接触しないようにゴム手袋，ビニール手袋，予防クリームで予防する必要がある（推奨度B～C1）。原因物質の特定ができないとき，もしくは特定されても職業性皮膚炎など原因物質が職場にあり排除できないときは産業医に連絡し場合によって職場の配置転換も考慮するが，改善しないときにはステロイド内服療法，免疫抑制薬（推奨度C1），紫外線療法（推奨度A，B）が必要とな

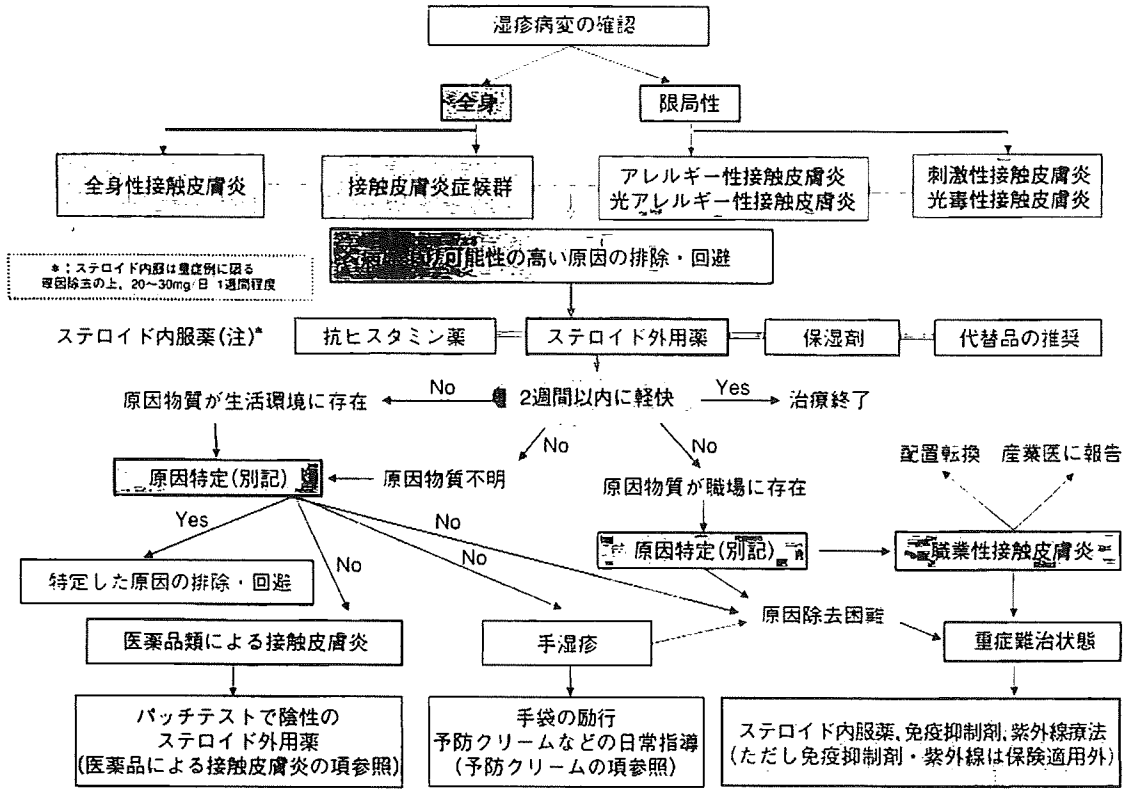

図4　接触皮膚炎診断アルゴリズム（文献1）から引用）

る。歯科金属などの金属が原因と考えられる全身性接触皮膚炎では金属ダイエット，歯科金属除去（推奨度C1），クロモグリク酸ナトリウム内服（推奨度C1）が有効なことがある。

患者さんへの説明

陽性反応が現在の皮膚炎の原因であると確認できた場合，原因物質の性質，それが含まれる製品などの情報を伝える。また，それらの抗原が交叉反応を呈するものであれば，その情報についても伝える。

パッチテストにより，強刺激物質や腐食性化学物質は強い反応を生じることがある。また，色素沈着や色素脱失を起こしたり，まれには瘢痕を形成することがあり，強感作物質はパッチテストにより新たな感作を起こすことがある。未知の物質については，腐食性，刺激性，感作性，経皮吸収後の身体への影響などを確認し，安全性を明らかにしてから適切な濃度・基剤を設定した上でパッチテストを実施する。検査を実施する前には，上記のパッチテストの危険性を十分説明し，患者が同意した上で検査を行う。

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接触皮膚炎

松永佳世子

ポイント

★接触皮膚炎は海綿状態を伴う湿疹反応を症状とする．

★刺激性接触皮膚炎とアレルギー性接触皮膚炎，光が関与する光毒性接触皮膚炎と光アレルギー性接触皮膚炎がある．

★診断は，詳しい問診，痒み・痛み，皮疹の性状，部位などの身体所見で原因を推定し，パッチテストで原因物質を確定する．

★治療は，原因物質を避けること，代替品を選択すること，職業性では防御方法の指導を行い，炎症にはステロイド外用，重症では内服，抗ヒスタミン薬内服，そして刺激皮膚炎の予防には保湿薬の外用を行う．

はじめに

接触皮膚炎は日常診療でしばしば遭遇する疾患のひとつである．接触皮膚炎は外から皮膚に接触した化学物質によって炎症を起こすために，原因を明らかにし除去することができれば，完治できる疾患である．したがって，原因を的確に診断し除去すること，適切な代替品を選択すること，職業性接触皮膚炎では，防御方法を指導することが重要である．炎症症状には，ステロイド外用薬の塗布，痒みには抗アレルギー薬の内服，重症例ではステロイドの内服薬を短期間使用する．

接触皮膚炎を正しく診断する(図1)

接触皮膚炎は原因となる化学物質が外から皮膚に接触した後に，皮膚に痒みや痛みを起こさせ，紅斑，腫脹，丘疹，びらんなどの炎症を起こす疾患である．接触皮膚炎を正しく診断するには，詳しい問診を行う．どの部位から始まったか，痒いか(アレルギー性)，痛いか(刺激性)，どんな時に悪くなるか，日光曝露部位か，職業・趣味，化粧品や日用品の使用状況，そして忘れてはいけないのは外用薬による接触皮膚炎である．このような問診のあと，皮膚の症状を観る．全身に分布しているか，どの部位に強い症状があるか，露出部にあるか，痒みが強くて紅斑や漿液性丘疹が主体か(アレルギー性)，痛くて紅斑・鱗屑・大水疱など(刺激性)，慢性なら，苔癬化がみられる．以上の問診からアレルギー性機序が考えられるときはパッチテストを行う．光の関与が疑われるときは，光パッチテストを行う．パッチテストについては別稿で詳しく述べる．原因を推定するには部位から(図2)推定し，またアレルゲンの種類からも推定する(図3)．

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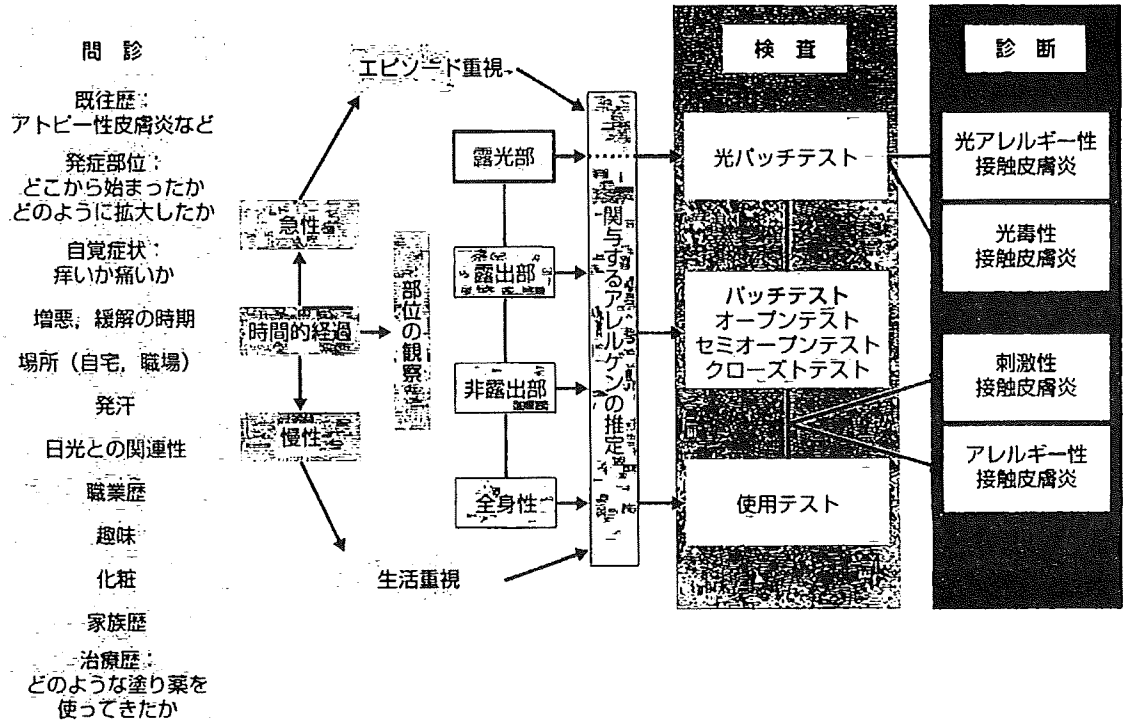
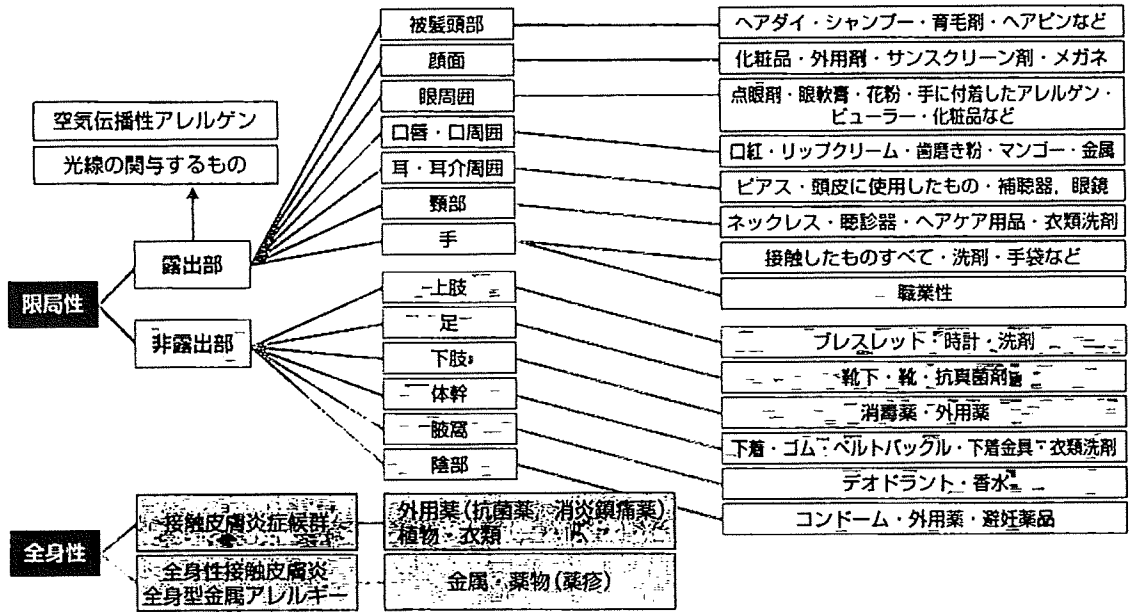
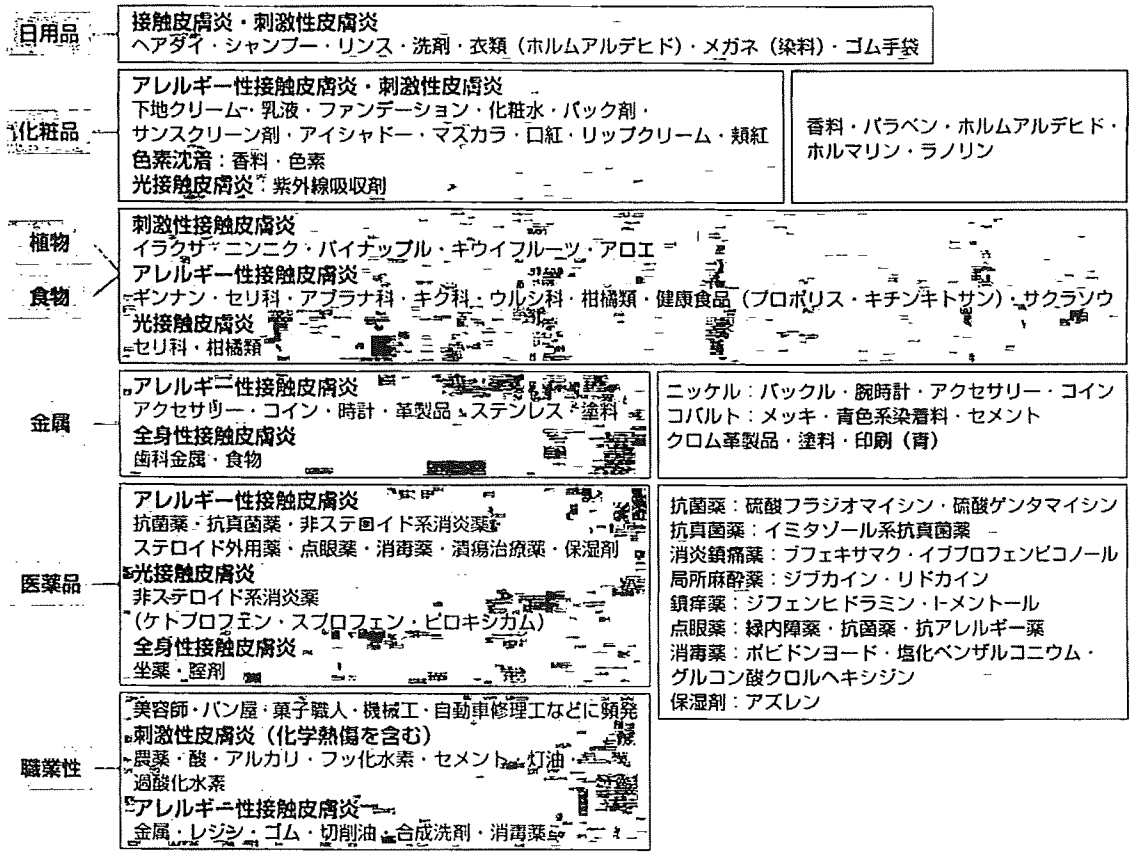


【図 1】 診断の手順（文献 1 から引用一部改変）



【図 2】 発症部位からアレルゲンを推定する（文献 1 から引用一部改変）



【図3】 原因の種類や職業などによって原因アレルゲンや刺激物質を考える（文献1から引用一部改変）

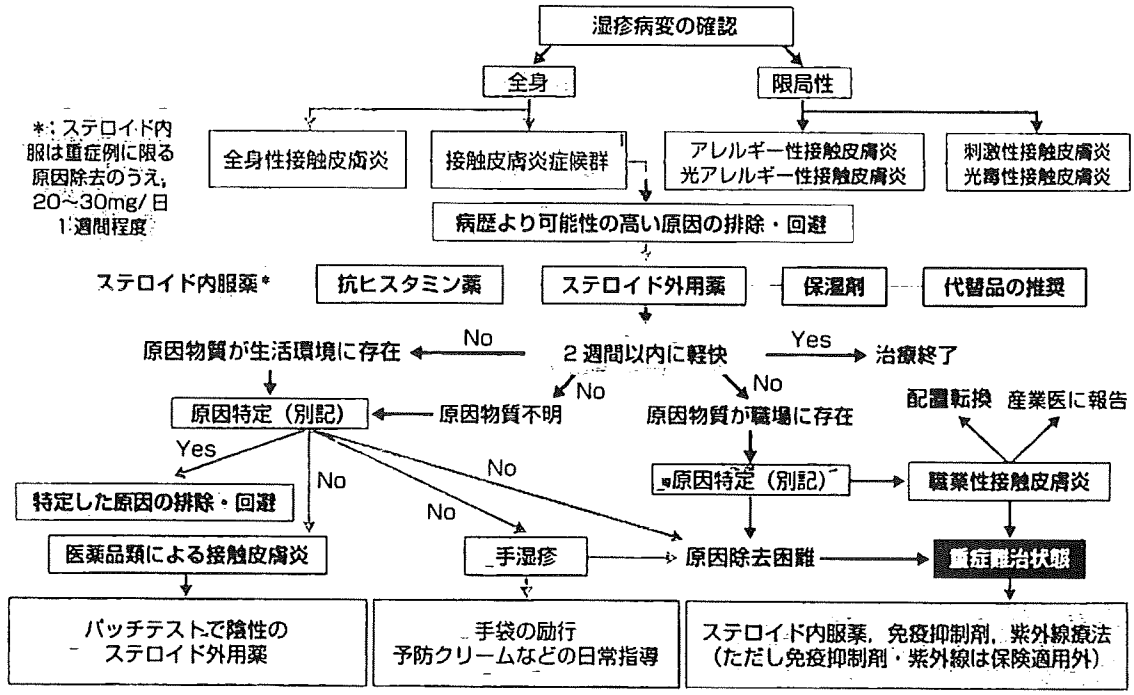
接触皮膚炎の病態を理解する

接触皮膚炎には，刺激性接触皮膚炎とアレルギー性接触皮膚炎がある．そして，接触した化学物質に光が当たって炎症を生じる接触皮膚炎のなかに，光毒性接触皮膚炎と光アレルギー性接触皮膚炎がある．

刺激性接触皮膚炎は，刺激を起こす化学物質が濃い濃度で皮膚に付くとだれにでも発症する．化学物質が皮膚の角化細胞の膜あるいは代謝を障害しサイトカイン，ケモカインの産生を誘導し，炎症細胞が浸潤し炎症が起こる．

アレルギー性接触皮膚炎は通常分子量1000未満の微量のハプテンが皮膚に侵入し，蛋白質と結合後，抗原提示細胞がこれを捕獲し所属リンパ節に遊走し，抗原情報をTリンパ球に伝えて感作リンパ球ができる．感作が成立した個体に再び抗原が皮膚に接触すると種々のサイトカイン，ケモカインの産生がみられ，肥満細胞の脱顆粒，血管拡張と内皮細胞の活性化，好中球，好酸球が浸潤し，続いてTリンパ球が浸潤し，海綿状態を生じる．

光毒性接触皮膚炎では物質に紫外線が当たり活性酸素が発生し組織や細胞傷害を起こす．光アレルギー性接触皮膚炎では皮膚に接触した物質に紫外線が当たり抗原提示細胞が光ハプテン修飾を受けてアレルギー反応が生じる．



【図4】 治療のアルゴリズム（文献1から引用）

接触皮膚炎の治療（図4）

接触皮膚炎では原因物質を確定し，除去することが第一である．炎症症状が重度あるいは全身に及ぶ場合は積極的にステロイドを全身的に使用するが，それ以外は，抗ヒスタミン薬内服，ステロイド外用で治療し，皮膚バリア機能障害・乾燥肌には治療と再発予防のために保湿剤を使用させ，また，代替品の選択を行い，再発防止に努める．外用薬が原因の接触皮膚炎ではパッチテスト陰性の外用薬で治療を行うことが重要で，硫酸フラジオマイシンや硫酸ゲンタマイシンの接触皮膚炎がしばしば認められるために，これを含まない外用薬で治療することが必要となる．

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Evaluation of changes of cell-surface thiols as a new biomarker for *in vitro* sensitization test

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ABSTRACT

In order to find a novel biomarker for a simple assay to predict skin sensitization, we evaluated cell-surface thiols as a biomarker reflecting intracellular signaling in THP-1 cells (human monocytic cell line). First, we found that a decrease of cell-surface thiols on hapten-treated THP-1 cells was induced in parallel with phosphorylation of p38 MAPK. Next, we confirmed that 2-mercaptoethanol in the culture medium and the differentiation state of THP-1 cells did not affect the changes of cell-surface thiols by hapten. Changes of cell-surface thiols on THP-1 cells were detected after 2 h treatment with most allergens (e.g., DNCB, $NiSO_4$), as well as some non-allergens (e.g., Tween80, benzalkonium chloride), though other non-allergens (e.g., SDS, glycerol) had no effect. When either a significant decrease or increase of cell-surface thiols (more than 15% in each case) was used as a criterion, the results using 36 allergens and 16 non-allergens were in good accordance with those of *in vivo* assays. Finally, we confirmed that ATP, which is released as a consequence of cytotoxicity, did not affect the changes of cell-surface thiols. Our results suggest that changes of cell-surface thiols may be useful for an *in vitro* sensitization assay, which we designate as the SH test.

1. Introduction

Predictive testing for ability to induce allergic contact dermatitis is a major part of the safety assessment of new ingredients in cosmetics and drugs to be applied topically. Animal model tests, such as the guinea pig maximization test (Magnusson and Kligman, 1969), have been employed as standard procedures for this purpose for many years. More recently, the murine local lymph node assay (LLNA) was adopted by the Organization for Economic Cooperation and Development (OECD) for skin sensitization hazard assessment (OECD Test Guideline 429). However, the development of alternative *in vitro* skin sensitization assays that do not use animals is now required, based on the 7th amendment of the European Council directive published in 2003.

It is known that in the induction phase of skin sensitization, antigen-presenting cells (APCs), including dendritic cells (DCs), migrate into lymph nodes and present antigens to T cells after having been activated by haptens. DCs, including Langerhans cells (LCs), are potent APCs and play an important role in the induction phase of skin sensitization by simple chemicals (Aiba and Tagami, 1999). As regards the activation of DCs, Aiba et al. demonstrated *in vitro* that human monocyte-derived DCs (MoDCs) responded to haptens such as nickel chloride ($NiCl_2$) and 2,4-dinitrochlorobenzene (DNCB) by significantly augmenting their expression of CD86, CD54 and HLA-DR, CCR7 and by increasing their production of pro-inflammatory cytokines (Aiba et al., 1997; Boisleve et al., 2004).

These findings suggested that $NiCl_2$ and DNCB induced the activation of signal transduction. Subsequently, it was reported that DNCB and Ni salts ($NiCl_2$ or $NiSO_4$) induced phosphorylation of p38 mitogen-activated protein kinase (MAPK), and that the augmentation of CD86, CD80 and CD83 induced by Ni salts is partially suppressed by a p38 MAPK inhibitor in MoDCs (Arrighi et al., 2001, 2003). The MAPKs are an important group of serine/threonine signaling kinases that, by modulating the phosphorylation and hence activation status of transcription factors, link transmembrane signaling with gene induction events in the nucleus. Therefore, phosphorylation of MAPKs is thought to reflect the activation of intracellular signaling.

On the basis of the above findings, phenotypic change and intracellular signaling of hapten-treated DCs can be considered as candidate biomarkers for developing non-animal alternative methods for skin sensitization testing, although it would be important to select an appropriate indicator cell line. However, it is difficult to use DCs as indicator cells in practical applications, because of donor-to-donor variability and limited availability of human blood for routine use. In order to overcome these problems, *in vitro* sensitization methods using cell lines such as THP-1, U-937 (Ashikaga et al., 2006; Sakaguchi et al., 2006; Python et al., 2007) and

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MUTZ-3 (Azam et al., 2006) have been reported. In these methods, the expression of surface biomarkers, such as CD86 and CD54, is measured by flow cytometry after treatment of the cells with haptens, such as DNCB and $NiSO_4$.

On the other hand, Mizuashi et al. reported that p38 MAPK plays crucial rule in the augmentation of CD86 expression on dendritic cells and that intracellular redox imbalance, which is a part of the up-stream signaling pathway of p38 MAPK, is very important for activation of MoDCs and THP-1 cells treated with haptens (Mizuashi et al., 2005). Moreover, Filomeni et al. reported that oxidation of exofacial membrane thiol groups by exogenous non-permeable oxidative form glutathione (GSSG) triggered a decrease of intracellular glutathione (GSH), phosphorylation of p38 MAPK, and apoptosis in U-937 cell lines (Filomeni et al., 2003). Further, we have recently found that cell-surface thiols play a role in the activation of MoDCs and THP-1 cells by haptens (Kagatani et al., submitted for publication; Hirota et al., 2009). In the present work, we used THP-1 cells as a replacement for DCs and evaluated the change of cell-surface thiols on hapten-treated cells as a candidate biomarker for an *in vitro* sensitization test.

2. Materials and methods

2.1. Chemicals

Oxazolone, *p*-benzoquinone, DNCB, methylchloroisothiazolinone/methylisothiazolinone (act. 1.5%) (MCI/MI), diphenylcyclopropenone (DPCP), *p*PD, potassium dichromate, maleic anhydride, 2-nitro-1,4-phenylenediamine (NPD), glutaraldehyde (act. 50%), benzoyl peroxide, propyl gallate, formaldehyde solution (act. 37%), cobalt sulfate heptahydrate ($CoSO_4$), ammonium tetrachloroplatinate ($(NH_4)_2PtCl_4$), isoeugenol, cinnamaldehyde, nickel sulfate hexahydrate ($NiSO_4$), ethylene diamine, phenylacetaldehyde, hexylcinnamic aldehyde (HCA), 2-mercaptobenzothiazole (2-MBT), eugenol, abietic acid, citral, benzocaine, geraniol, linalool, butylglycidylether, penicillin G, pyridine, thimerosal, Tween80, lactic acid (LA), glycerol, resorcinol, sodium dodecyl sulfate (SDS), benzalkonium chloride (BC), zinc chloride ($ZnCl_2$), dextran, glucose and Ionomycin were purchased from Sigma–Aldrich Corporation (St. Louis, MI). Manganese chloride tetrahydrate ($MnCl_2$), methyl salicylate (MS), octanoic acid, 2-propanol, propylene glycol, xylene, saccharin, 6-methyl coumarin (6-MC) and adenosine triphosphate (ATP) were purchased from Wako Pure Chemicals (Osaka, Japan). Dimethyl sulfoxide (DMSO) was purchased from Kanto Chemical (Tokyo, Japan), squaric acid di-*n*-butyl ester (SADBE) and 2,4,6-trinitrochlorobenzene (TNCB) from Tokyo Kasei Kogyo (Tokyo, Japan). Non-permeable thiol-reactive compound Alexa Fluor C5 maleimide (AFM) was purchased from Invitrogen Life Technologies (Carlsbad, CA). The following monoclonal antibodies (mAbs); FITC-conjugated anti-CD86, PE-conjugated CD83, PE-conjugated anti-phospho-p38 MAPK (T180/Y182), FITC- and PE-conjugated mouse IgG1 mAbs were purchased from BD-PharMingen (San Diego, CA). Recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF) was purchased from PeproTech EC (London, UK). Recombinant interleukin-4 (rhIL-4) and tumor necrosis factor-α (rhTNF-α) were purchased from R&D systems (Minneapolis, MN).

2.2. Cells and culture

THP-1 cells were obtained from the American Type Culture Collection (Rockville, MD). These cells were maintained in RPMI 1640 medium (Invitrogen Life Technologies) with 1% (v/v) antibiotic–antimycotic (Invitrogen Life Technologies), 10% FBS (v/v) (JRH Biosciences, Lenexa, KS) and appropriate concentration of 2-mercaptoethanol (2-ME) (Invitrogen Life Technologies) at 37 °C in a 5% CO_2 incubator.

2.3. Generation of mature DCs from THP-1 cell lines

Preparation of mature DCs from THP-1 cells was performed as described by Berges et al. (2005). In brief, culturing of THP-1 cells for 24 h in serum-free medium supplemented with 1500 IU/mL rhGM-CSF, 1000–2000 IU/mL rhTNF-α 200 ng/mL ionomycin, and 3000 IU/mL IL-4 resulted in complete differentiation of the cells into mature DCs with characteristic stellate morphology. The maturity of the DCs were checked by measuring expression of CD86 and CD83 by flow cytometry with an FACSCalibur CellQuest (Becton Dickinson, San Jose, CA).

2.4. Chemical treatment of THP-1 cells

Doses of chemicals were set at sub-toxic levels based on the IC_{50} values in MTT assay (Mossmann, 1983). In brief, 0.1 mL of THP-1 cell suspension (1×10^6 cells/mL) was seeded in 96-well plates and cultured with test chemicals at various concentrations for 2 h. Cells were washed twice and 100 μL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution (0.52 mg/mL, dissolved in the medium) was applied to each well. After reaction for 3 h at 37 °C, the absorbance was measured at 570 nm and 660 nm and IC_{50} (μg/mL) values for the test chemicals were evaluated. The test doses were then set at concentration equivalent to the IC_{50} value, and one-third and one-ninth of the IC_{50} value. If the IC_{50} value could not be estimated, the test doses were set as the maximum concentration of 5000 μg/mL (for water-soluble chemicals) or 1000 μg/mL (for water-insoluble chemicals), and one-third and one-ninth of the maximum (Sakaguchi et al., 2006).

2.5. Analysis of phospho-p38 MAPK by flow cytometry

At 2 h after treatment with chemicals, the intracellular expression of phospho-p38 MAPK (pp38 MAPK) was analyzed by flow cytometry. Cells were permeabilized with Intraprep (Beckman Coulter) according to the manufacturer's protocol, and cell staining was performed using PE-conjugated anti-pp38 MAPK (T180/Y182) (20 μL/1 × 10^6 cells). The cells were then washed with PBS supplemented with 0.1% BSA (FACS buffer), and analyzed by flow cytometry (EPICS XL-MCL System II (Beckman Coulter)). Relative fluorescence intensity (RFI) was calculated from the mean fluorescence intensity (MFI) by use of the following formula: RFI (% of control) = (MFI of chemical-treated cells/MFI of vehicle control cells) × 100.

2.6. Analysis of cell-surface thiols by flow cytometry

THP-1 cells were seeded at 1×10^6 cells/mL. Lipophilic compounds were dissolved in DMSO and added to the culture medium. After 2 h treatment with chemicals, THP-1 cells were recovered, washed twice with PBS, then incubated with 100 μL of Alexa Fluor 488 C5 maleimide (AFM) (10 μM) PBS solution for 30 min, 37 °C. After having been washed again with PBS, cells were analyzed by flow cytometry with an EPICS XL-MCL System II (Beckman Coulter, Fullerton, CA). Dead cells were gated out by staining with propidium iodide (PI, 0.625 μg/ml). A total of 10,000 living cells was analyzed. When the cell viability was less than 50%, relative fluorescence intensity (RFI) was not calculated from mean fluorescence intensity (MFI) because of diffuse labeling of cytoplasmic structures due to cell membrane destruction (Becker et al., 1994).

RFI was calculated by use of the following formula: RFI (% of control) = (MFI of chemical-treated cells/MFI of vehicle control cells) × 100.

2.7. Measurement of extracellular ATP concentration released from chemical-treated THP-1 cells

THP-1 cells were treated with chemicals for 10, 30, 60 and 120 min. Culture supernatants were recovered and ATP was measured using a CellTiter-Glo™ Assay kit according to the manufacturer's recommendation (Promega, Madison, WI).

2.8. Statistical analysis

The statistical significance of differences in the RFI of cell-surface thiols and phosphorylated p38 MAPK between non-treated THP-1 cells and chemical-treated THP-1 cells was evaluated by using the paired Student's *t*-test (Snedecor and Cochan, 1989). The correlation coefficient (*r* value) was determined in this paper by the use of Pearson's correlation statistics (Snedecor and Cochan, 1989).

3. Results

3.1. Changes of cell-surface thiols and phosphorylation of p38 MAPK in chemical-treated THP-1 cells

THP-1 cells were treated with various chemicals, and the changes of cell-surface thiols and phosphorylation of p38 MAPK were examined by flow cytometry. Dose setting and the IC_{50} values

Table 1
C_{50} value of test chemicals.

Test samples		IC_{50} (mg/mL)	Solvent	References
ensitizers				
)xazolone	–	783	DMSO	National Toxicology Program (1999)
)-Benzoquinone		15	DMSO	National Toxicology Program (1999)
)NCB	2,4-Dinitrochlorobenzene	158	DMSO	National Toxicology Program (1999)
NCB	2,4,6-Trinitrochlorobenzene	56	DMSO	
ICI/MI	Methyl chloroisothiazolinone/methylisothiazolinone (act. 1.5%)	385	Saline	National Toxicology Program (1999)
)PCP	Diphenylcyclopropenone	63	DMSO	Gerberick et al. (2005)
PD	*p*-Phenylenediamine	>5000	Medium	National Toxicology Program (1999)
otassium dichromate	–	462	Saline	National Toxicology Program (1999)
Maleic anhydride		1796	Saline	
IPD	2-Nitro-1,4-phenylenediamine	5000	Medium	Gerberick et al. (2005)
lutaraldehyde (act. 50%)	–	157	Saline	Gerberick et al. (2005)
ADBE	Squaric acid di-*n*-butyl ester	650	DMSO	Manome et al. (1999)
enzoyl peroxide	–	100	DMSO	National Toxicology Program (1999)
ropyl gallate	–	>1000	DMSO	National Toxicology Program (1999)
ormaldehyde	Formaldehyde solution (act. 37%)	190	Saline	National Toxicology Program (1999)
oSO_4	Cobalt sulfate heptahydrate	2222	Saline	National Toxicology Program (1999)
$NH_4)_2PtCl_4$	Ammonium tetrachloroplatinate	2934	Medium	National Toxicology Program (1999)
oeugenol	–	399	DMSO	National Toxicology Program (1999)
A	Cinnamaldehyde	470	DMSO	National Toxicology Program (1999)
iSO_4	Nickel sulfate hexahydrate	>5000	Medium	National Toxicology Program (1999)
thylene diamine	–	1439	Saline	National Toxicology Program (1999)
henylacetaldehyde	–	115	DMSO	Gerberick et al. (2005)
esorcinol	–	4955	Medium	Basketter et al. (2007)
CA	Hexylcinnamic aldehyde	155	DMSO	National Toxicology Program (1999)
-MBT	2-Mercaptobenzothiazole	>1000	DMSO	National Toxicology Program (1999)
ugenol	–	703	DMSO	National Toxicology Program (1999)
bietic acid	–	333	DMSO	National Toxicology Program (1999)
tral	–	560	DMSO	National Toxicology Program (1999)
enzocaine	–	>1000	DMSO	National Toxicology Program (1999)
eraniol	–	743	DMSO	Gerberick et al. (2005)
nalool	–	>1000	DMSO	Gerberick et al. (2005)
utylglycidylether	–	>1000	DMSO	National Toxicology Program (1999)
nicillin G	–	>5000	Medium	National Toxicology Program (1999)
ridine	–	>5000	Medium	National Toxicology Program (1999)
nCl_2	Manganese chloride	3000	Medium	Mizuashi et al. (2005)
iimerosal	–	17	Saline	Mizuashi et al. (2005)
m-sensitizers				
S	Methyl salicylate	>1000	DMSO	National Toxicology Program (1999)
veen80	–	>5000	Medium	National Toxicology Program (1999)
	Lactic acid	3350	Medium	National Toxicology Program (1999)
ycerol	–	>5000	Medium	Gerberick et al. (2005)
S	Sodium dodecyl sulfate	50	Saline	National Toxicology Program (1999)
	Benzalkonium chloride	2	Saline	National Toxicology Program (1999)
Cl_2	Zinc chloride	1472	Saline	National Toxicology Program (1999)
xtran	–	>5000	Medium	National Toxicology Program (1999)
ucose	–	>5000	Medium	
tanoic acid	–	>1000	DMSO	Gerberick et al. (2005)
Propanol	–	>5000	Medium	National Toxicology Program (1999)
pylene glycol	–	>5000	Medium	National Toxicology Program (1999)
lene	–	>1000	DMSO	National Toxicology Program (1999)
charin	–	>1000	DMSO	Gerberick et al. (2005)
MC	6-Methyl coumarin	>1000	DMSO	National Toxicology Program (1999)
MSO	Dimethyl sulfoxide	>5000	Medium	Gerberick et al. (2005), Sakaguchi et al. (2006)

$_{50}$ values were determined by means of MTT assay (Mossmann, 1983). Chemicals were dissolved in a solvent such as DMSO, saline or culture medium, and added to the ture medium. If the dose giving 50% cell viability (IC_{50}) could not be estimated, the maximum concentration was set as 5000 μg/mL (for water-soluble chemicals) or 00 μg/mL (for water-insoluble chemicals).

of test chemicals are summarized in Table 1. Skin sensitizers such as DNCB, SADBE, formalin, CA and 2-MBT induced both phosphorylation of p38 MAPK and decrease of cell-surface thiols within 2 h after stimulation, while non-sensitizers, such as LA and SDS, did not induce p38 MAPK phosphorylation or decrease of cell-surface thiols (Fig. 1A and B). Furthermore, we found a good correlation

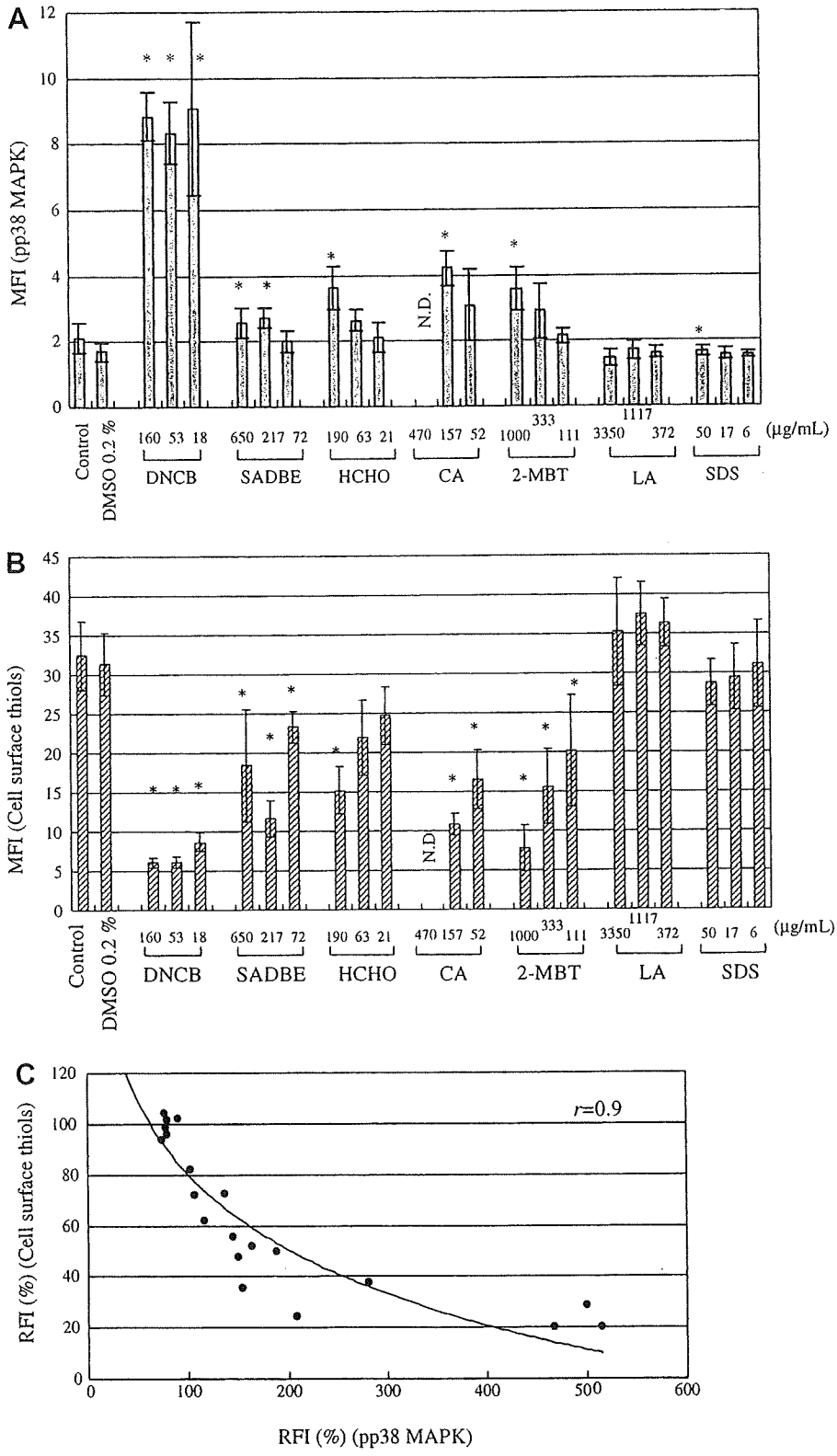

Fig. 1. Phosphorylation of p38 MAPK and changes of cell-surface thiols in chemical-treated THP-1 cells. Cells were treated with three concentrations (IC_{50}, one-third of IC_{50}, and one-ninth of IC_{50}) of each chemical for 2 h. After treatment, (A) phosphorylated p38 MAPK and (B) cell-surface thiols were measured by flow cytometry. Mean values of MFI ± SD for at least three independent experiments are shown: *; $p < 0.05$ and ND; not determined. (C) Correlation between the level of p38 MAPK phosphorylation and the decrease of cell-surface thiols in THP-1 cells treated with chemicals. RFI values were determined as described in Section 2. The value of the correlation coefficient (r value) was 0.9.

($r = 0.9$) between the level of p38 MAPK phosphorylation and the decrease of cell-surface thiols in chemical-treated THP-1 cells, as shown in Fig. 1C.

3.2. Effect of 2-ME in the culture medium on cell-surface thiols

In general, 2-ME is added to the culture medium for maintenance of cell growth, and as it is a reducing agent, it may influence cell-surface thiols. However, as shown in Fig. 2, the concentration of 2-ME in the culture medium had no effect on the change of cell-surface thiols on chemical-treated THP-1 cells.

3.3. Effect of differentiation state of THP-1 cells on changes of cell-surface thiols

Maturation of DCs is accompanied with phenotypic changes, such as enhanced CD antigen expression. Therefore, we examined whether maturation of THP-1 affects hapten-induced changes of cell-surface thiols. We found that maturation resulted in an increase of cell-surface thiols (Fig. 3B), as well as augmentation of CD86 and CD83 expression, as shown in Fig. 3A. However, a comparison of mature DCs derived from THP-1 cells and naïve THP-1 cells indicated that maturation of THP-1 cells did not greatly affect the changes of cell-surface thiols induced by DNCB treatment (Fig. 3C).

3.4. Suitability of changes of cell-surface thiols as a biomarker for in vitro sensitization test

Based on the above results, we chose an appropriate concentration of 2-ME for the culture medium, and suitable cell differentiation conditions for THP-1 cells. To further examine the suitability of changes of cell-surface thiols as a candidate sensitization biomarker, we measured the changes of cell-surface thiols in response to a wide range of chemicals (52 chemicals, including skin sensitizers and non-sensitizers) by means of flow cytometry (Table 2). Most of the skin sensitizers caused a decrease of cell-surface thiols at concentrations in the range giving 50–100% cell viability. On the other hand, skin sensitizers, such as DPCP, pyridine and $MnCl_2$, caused an increase of cell-surface thiols. When we adopted the tentative criterion that a decrease of cell-surface thiols to less than 85% (control; 100%) was a significant change, contact sensitizers such as oxazolone, *p*-benzoquinone, MCI/MI, DNCB, TNCB, *p*PD, potassium dichromate, maleic anhydride, NPD, glutaraldehyde, SADBE, benzoyl peroxide, propyl gallate, formaldehyde, $CoSO_4$, $(NH_4)_2PtCl_4$, isoeugenol, cinnamaldehyde, $NiSO_4$, ethylenediamine, phenylacetaldehyde, resorcinol, HCA, 2-MBT, eugenol, abietic acid, citral, benzocaine, geraniol, linalool, butylglycidylether and thimerosal, and non-sensitizers, such as Tween80, BC, $ZnCl_2$ and 6-MC, were judged as positive, while other skin sensitizers, such as DPCP, penicillin G, pyridine and $MnCl_2$, and non-sensitizers, such as MS, lactic acid, glycerol, SDS, dextran, glucose, octanoic acid, 2-propanol, propylene glycol, xylene, saccharin and DMSO, were judged as negative. The results of *in vivo* data (National Toxicology Program, 1999; Manome et al., 1999; Mizuashi et al., 2005; Gerberick et al., 2005; Sakaguchi et al., 2006; Basketter et al., 2007) and *in vitro* assays measuring cell-surface thiols on THP-1 cells (this study) are compared in Table 3. On the basis of the tentative criterion, the agreement between *in vivo* assays and decrease of cell-surface thiols was 85%. If we employed the criterion that either a decrease of cell-surface thiols to less than 85% (control; 100%) or an increase of cell-surface thiols to more than 115% was a significant change, three contact sensitizers, DPCP, pyridine and $MnCl_2$, previously judged as negative became positive. This improved the rate of agreement with *in vivo* assays to 90%.

3.5. Effect of ATP on cell-surface thiols

We investigated changes of cell-surface thiols on THP-1 cells in the presence of ATP or chemicals that induce the release of ATP from THP-1 cells, because it was not known whether cell-surface thiols would be affected by mediators released from lysed cells. As shown in Fig. 4A and B, the tested concentrations of ATP did not affect cell-surface thiols, and ATP release from the cells was no more than 1 μM following treatment with various chemicals.

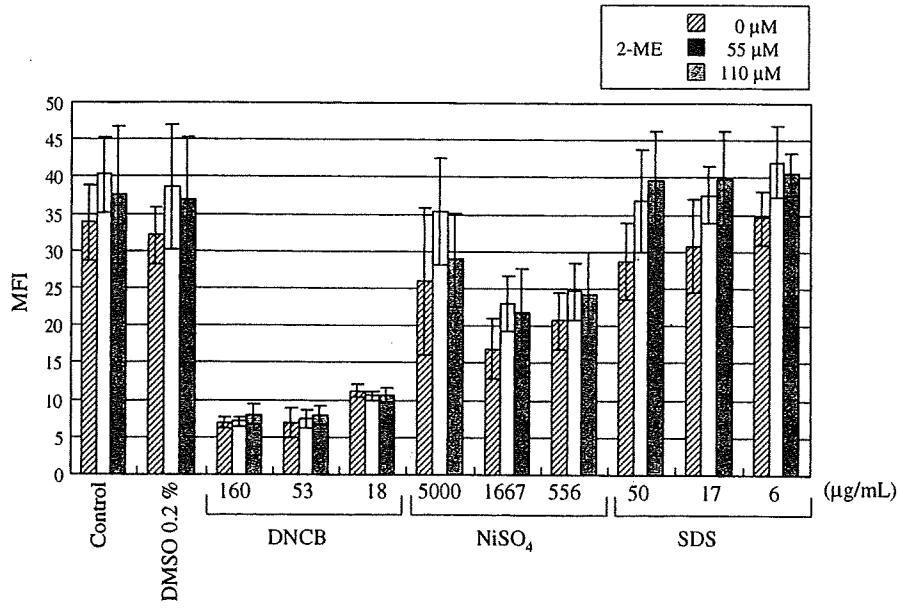

Fig. 2. Effect of 2-mercaptoethanol (2-ME) concentration in the culture medium on changes of cell-surface thiols on chemical-treated THP-1 cells. THP-1 cells were cultured in culture medium supplemented with 55 μM 2-ME, 110 μM 2-ME, or 0 μM 2-ME. After treatment with DNCB, $NiSO_4$ or SDS for 2 h, cell-surface thiols were measured by flow cytometry. Mean values of MFI ± SD for at five independent experiments are shown.

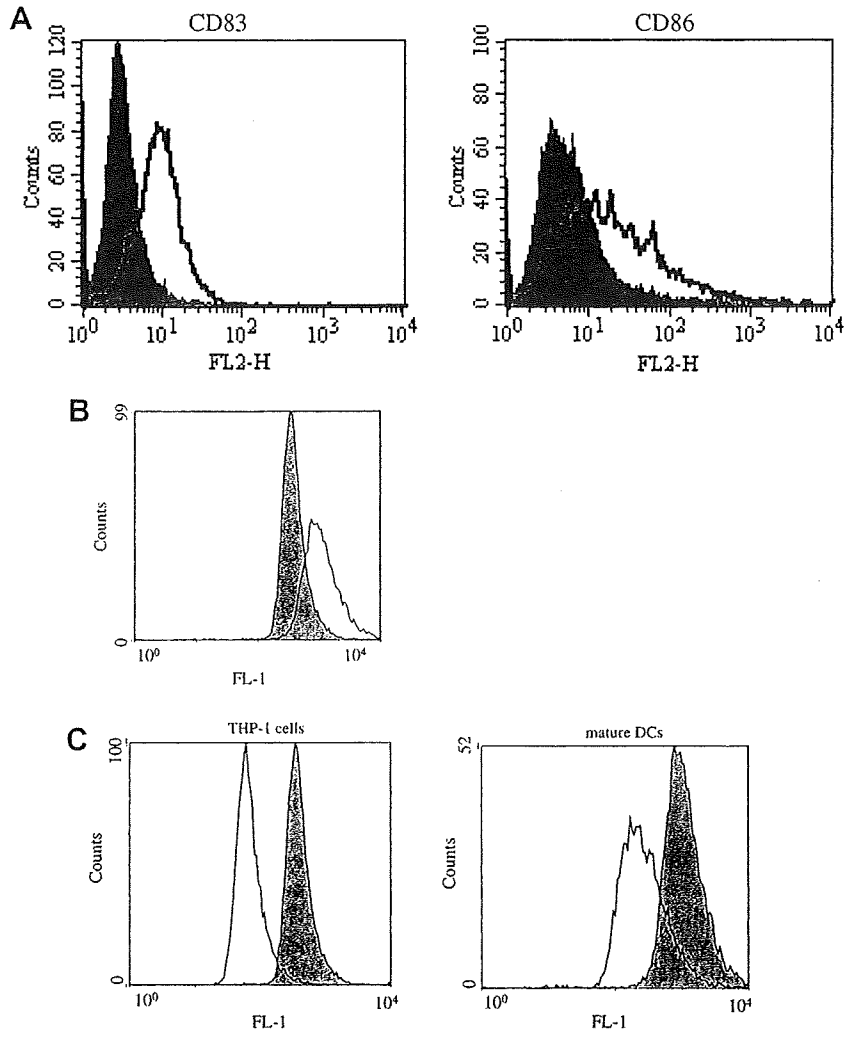

Fig. 3. Effect of differentiation state on changes of cell-surface thiols on chemical-treated THP-1 cells. Flow cytometric analysis of (A) CD86 and CD83 expression, (B) cell-surface thiols on THP-1 cells (filled histograms) and mature DCs derived from THP-1 cells (open histograms), and (C) changes of cell-surface thiols on THP-1 cells and mature DCs derived from THP-1 cells treated with 160 μg/mL DNCB. In (C), filled histograms indicate the solvent control and open histograms, cells treated with 160 μg/mL DNCB. Representative results of three independent experiments are shown.

4. Discussion

In order to develop alternative *in vitro* skin sensitization assays that do not use animals, *in vitro* sensitization tests based on phenotypic changes, such as CD86 expression, of hapten-treated DCs and cell lines have been proposed (Aiba et al., 1997; Sakaguchi et al., 2006). On the other hands, intracellular redox imbalance plays a key role in the regulation of phenotypic changes in dendritic cells and THP-1 cells, and could be suitable as a biomarker for an *in vitro* sensitization test (Aiba et al., 2003; Mizuashi et al., 2005). It has been suggested that exofacial membrane thiol groups play a role as sensors of cell activation (Nakashima et al., 2002; Filomeni et al., 2003). The measurement of change of cell-surface thiols is easy and takes only a short time (several hours). In the present work, we attempted to evaluate the suitability of changes of cell-surface thiols as a biomarker for an *in vitro* sensitization test. For this purpose, we employed a non-permeable thiol-reactive compound, Alexa Fluor 488 C5 maleimide (AFM), which we considered would be suitable to detect changes of cell-surface thiols induced by binding of haptens to cell-surface proteins, oxidation of cell-surface thiols, and so on.

First, we examined the relationship between phosphorylation of p38 MAPK and changes of cell-surface thiols on chemical-treated THP-1 cells. It was reported that high affinity of a small reactive chemical toward thiol groups is important for the activation of monocyte-derived dendritic cells and can support the process of sensitization (Becker et al., 2003). Our results and previous reports support the idea that cell-surface thiols are a potential biomarker reflecting intracellular signaling. Next, we examined suitable assay conditions. There are many factors that may influence the assay, but we paid particular attention to the concentration of 2-ME in the culture medium and the conditions for differentiation of THP-1 cells as the indicator cells. This was because 2-ME might directly affect the cell-surface thiols, not haptens as a reducing agent, and because expression of cell membrane proteins, such as CD antigens, in response to haptens was reported to change with cell differentiation (Berges et al., 2005). We found that neither of these factors greatly influences the changes of cell-surface thiols in response to haptens.

In general, the majority of haptens (or their metabolites) have electrophilic properties and are able to react with the side chains of many amino acids in proteins, including lysine and cysteine (Gerberick et al., 2004). Thus, we speculated that the decrease of cell-surface thiols by DNCB and $NiSO_4$ might be caused by binding of the haptens to cell membrane proteins. This idea was supported by the finding that ATP did not affect cell-surface thiols. ATP is

Table 2
Effect of test chemicals on THP-1 cell-surface thiols.

Test samples	RFI (%) (cell-surface thiols)			Cell-surface thiols
	IC_{50}	1/3 × IC_{50}	1/9 × IC_{50}	
Sensitizers				
Oxazolone	ND	42.8 ± 6.4*	103 ± 20	Decrease
p-Benzoquinone	ND	58.8 ± 18.5*	95.9 ± 11.4	Decrease
DNCB	20.9 ± 5.0*	20.1 ± 4.8*	28.3 ± 6.1*	Decrease
MCI/MI	37.7 ± 11.7*	81.2 ± 5.3*	100 ± 3.9	Decrease
TNCB	ND	22.8 ± 1.9*	32.4 ± 7.0*	Decrease
DPCP	ND	65.2 ± 37.7	563 ± 116*	Increase
*p*PD	32.3 ± 19.0*	44.5 ± 18.4*	77.1 ± 1.5*	Decrease
Potassium dichromate	61.0 ± 3.2*	71.1 ± 5.2*	74.6 ± 5.7*	Decrease
Maleic anhydride	63.4 ± 12.9*	85.5 ± 11.6	87.9 ± 7.2	Decrease
NPD	51.9 ± 15.4*	72.8 ± 4.3*	79.3 ± 8.1*	Decrease
Glutaraldehyde (act. 50%)	ND	87.4 ± 7.4	70.4 ± 3.4*	Decrease
SADBE	55.7 ± 15.9*	35.4 ± 3.8*	71.7 ± 5.7*	Decrease
Benzoyl peroxide	ND	70.4 ± 10.9*	91.2 ± 12.5	Decrease
Propyl gallate	56.7 ± 8.5*	66.3 ± 8.2*	68.5 ± 2.9*	Decrease
Formaldehyde	49.9 ± 4.8*	72.2 ± 14.3*	82.1 ± 15.5*	Decrease
$CoSO_4$	36.8 ± 16.7*	41.4 ± 17.8	47.2 ± 18.4	Decrease
$(NH_4)_2PtCl_4$	117 ± 11.3	60.7 ± 4.9*	58.9 ± 3.1*	Decrease
Isoeugenol	ND	77.3 ± 3.4*	90.0 ± 10.3	Decrease
CA	ND	33.9 ± 2.5*	51.7 ± 9.7*	Decrease
$NiSO_4$	79.8 ± 26.6	48.4 ± 10.1*	52.8 ± 5.7*	Decrease
Ethylene diamine	105 ± 20.8	80.5 ± 4.0*	87.1 ± 3.4	Decrease
Phenylacetaldehyde	45.2 ± 6.7*	79.0 ± 7.4*	105 ± 4.5	Decrease
Resorcinol	62.4 ± 10.5*	86.6 ± 7.1	86.1 ± 6.2	Decrease
HCA	74.2 ± 3.2*	71.0 ± 2.7*	86.8 ± 9.8	Decrease
2-MBT	20.5 ± 13.0*	39.3 ± 20.0*	49.0 ± 23.9*	Decrease
Eugenol	ND	83.8 ± 11.1*	90.6 ± 6.9	Decrease
Abietic acid	ND	84.4 ± 7.0*	107 ± 8.4	Decrease
Citral	ND	ND	59.3 ± 0.94*	Decrease
Benzocaine	79.3 ± 2.6*	89.2 ± 4.8	102 ± 6.6	Decrease
Geraniol	108 ± 30.2	74.5 ± 11.5*	78.9 ± 11.2*	Decrease
Linalool	75.2 ± 11.2	74.2 ± 3.4*	86.0 ± 4.2	Decrease
Butylglycidylether	80.1 ± 11.0*	99.7 ± 12.0	104 ± 10.4	Decrease
Penicillin G	96.5 ± 19.7	102 ± 10.1	99.1 ± 11.2	No-change
Pyridine	126 ± 1.3*	123 ± 5.6*	112 ± 5.3	Increase
$MnCl_2$	143 ± 23.0*	177 ± 14.4*	100 ± 15.0*	Increase
Thimerosal	53.8 ± 17.0*	76.1 ± 18.1	83.8 ± 8.6	Decrease
Non-sensitizers				
MS	113 ± 16.0	88.5 ± 22.1	102 ± 23.5	No-change
Tween80	61.1 ± 15.4*	77.7 ± 20.7	81.2 ± 18.1	Decrease
LA	97.9 ± 10.0	104 ± 7.7	103 ± 5.3	No-change
Glycerol	91.6 ± 1.1	91.0 ± 1.3	94.8 ± 1.6	No-change
SDS	93.4 ± 13.8	98.2 ± 16.5	104 ± 24.5	No-change
BC	82.8 ± 8.4*	90.8 ± 9.1	94.3 ± 11.4	Decrease
$ZnCl_2$	53.3 ± 13.1*	58.5 ± 11.3*	88.5 ± 20.6	Decrease
Dextran	103 ± 2.6	98.4 ± 1.2	97.8 ± 2.1	No-change
Glucose	92.3 ± 6.6	91.1 ± 6.4	93.4 ± 7.5	No-change
Octanoic acid	109 ± 7.4	103 ± 11.6	105 ± 9.8	No-change
2-Propanol	98.9 ± 18.4	95.7 ± 14.7	104 ± 12.8	No-change
Propylene glycol	105 ± 5.6	104 ± 7.1	93.1 ± 1.9	No-change
Xylene	95.4 ± 18.5	89.4 ± 9.4	91.4 ± 8.4	No-change
Saccharin	97.7 ± 7.0	95.6 ± 5.8	98.8 ± 6.3	No-change
6-MC	45.6 ± 7.2*	85.1 ± 10.3	88.9 ± 12.8	Decrease
DMSO	108 ± 6.7	111 ± 4.6	109 ± 1.0	No-change

Cells were treated with three concentrations (IC_{50}, one-third of IC_{50}, and one-ninth of IC_{50}) of each chemical shown in Table 1 for 2 h. After treatment, the changes of cell-surface thiols were measured by flow cytometry and RFIs were calculated. Mean values ± SD for at least three independent experiments are shown.
* $p < 0.05$.

thought to be a pro-inflammatory mediator that is released extracellularly as a consequence of cell lysis or damage. ATP released from chemically injured keratinocytes may lead to irritant dermatitis (Mizumoto et al., 2003), and ATP-sensitive $P2X_7$ receptor ($P2X_7R$) stimulates the maturation and release of interleukin 1β (IL-1β) in macrophages (Donnelly-Roberts et al., 2004).

High sensitivity, an easy detection procedure, and a short assay time are important requirements for a suitable biomarker for an *in vitro* sensitization test. Measurement of changes of cell-surface thiols meets these requirements, and our results (Table 3) indicate that these changes could indeed be used as a biomarker. However, some false positives (Tween80, BC, $ZnCl_2$ and 6-MC) were detected. Among them, $ZnCl_2$ was also positive in LLNA. Moreover, it is not known why a few haptens, such as DPCP, $MnCl_2$ and pyridine, caused an increase, not a decrease, of cell-surface thiols. A decrease of cell-surface thiols might be caused by binding of chemicals to cell-surface proteins and oxidation of cell-surface and intracellular proteins, because it was reported that intra- and extracellular redox states were related to each other (Moriarty-Craige and Jones, 2004). Furthermore, our data indicate a relationship between the level of p38 MAPK phosphorylation and the decrease of cell-surface thiols in chemical-treated THP-1 cells. Based on this result, we

Table 3
Relationship between *in vivo* sensitization tests and the present *in vitro* sensitization test measuring changes of cell-surface thiols as a biomarker.

Test samples	*In vivo* sensitization test			*In vitro* test (changes of cell-surface thiols)	
	GPMT/BT	LLNA	HMT	Criterion	
				RFI < 85 % ($p < 0.05$)	RFI < 85% ($p < 0.05$) and RFI > 115% ($p < 0.05$)
Sensitizer					
Oxazolone	+	+		+	+
p-Benzoquinone	+	+		+	+
MCI/MI	+	+		+	+
DNCB	+	+		+	+
TNCB				+	+
DPCP		+		–	+
*p*PD	+	+	+	+	+
Potassium dichromate	+	+	+	+	+
Maleic anhydride				+	+
NPD		+		+	+
Glutaraldehyde (act. 50%)		+		+	+
SADBE				+	+
Benzoyl peroxide	+	+		+	+
Propyl gallate	+	+		+	+
Formaldehyde	+	+	+	+	+
$CoSO_4$	+[a]	+[a]	+[a]	+	+
$(NH_4)_2PtCl_4$	+	+		+	+
Isoeugenol	+	+		+	+
CA	+	+	+	+	+
$NiSO_4$	+	–	+	+	+
Ethylene diamine	+	+		+	+
Phenylacetaldehyde		+		+	+
Resorcinol	–	+		+	+
HCA	+	+		+	+
2-MBT	+	+	+	+	+
Eugenol	+	+		+	+
Abietic acid	+	+		+	+
Citral	+	+	+	+	+
Benzocaine	+	+/–	+/–	+	+
Geraniol		+		+	+
Linalool		+		+	+
Butylglycidylether	+	+	+	+	+
Penicillin G	+	+	+	–	–
Pyridine		+		–	+
$MnCl_2$				–	+
Thimerosal				+	+
Non-sensitizer					
MS	–	–	–	–	–
Tween80	–	–		+	+
LA	–	–		–	–
Glycerol		–		–	–
SDS	–	+	–	–	–
BC	–	–		+	+
$ZnCl_2$		+[b]		+	+
Dextran	–	–		–	–
Glucose				–	–
Octanoic acid		–		–	–
2-Propanol	–	–		–	–
Propylene glycol	–	–		–	–
Xylene		+	–	–	–
Saccharin		–		–	–
6-MC	–	–	–	+	+
DMSO		+		–	–

GPMT/BT: guinea pig maximization test and Buhler test, LLNA: local lymph node assay, and HMT: human maximization test. Data are taken from the literature (National Toxicology Program, 1999; Gerberick et al., 2005; Basketter et al., 2007). (+) Positive and (–) Negative.

[a] Result for cobalt chloride.

[b] Result for zinc sulfate.

speculate that a decrease of cell-surface thiols reflects activation of intracellular signal transduction pathways. As for the increase of cell-surface thiols, a thiol antioxidant, *N*-acetyl-L-cysteine (NAC), was reported to increase cell-surface thiols on human peripheral blood mononuclear cells (PBMCs) by reducing membrane protein disulfides (Laragione et al., 2003). Moreover, it was demonstrated that dithiothreitol (DTT), which is a thiol reducing regent, induced phosphorylation of c-Jun N-terminal kinase (JNK) in response to endoplasmic reticulum stress (Urano et al., 2000). Though the mechanism of this change by skin sensitizers remains to be investigated, increase of cell-surface thiols may also reflect activation of intracellular signal transduction pathways in some cases.

In conclusion, we examined suitable assay conditions for measurement of changes of cell-surface thiols, and measured the changes of cell-surface thiols on THP-1 cells in response to various test chemicals. Our results indicate that changes of cell-surface thiols may be useful as a biomarker for an *in vitro* sensitization assay with low false positives, and we named this assay the SH test.

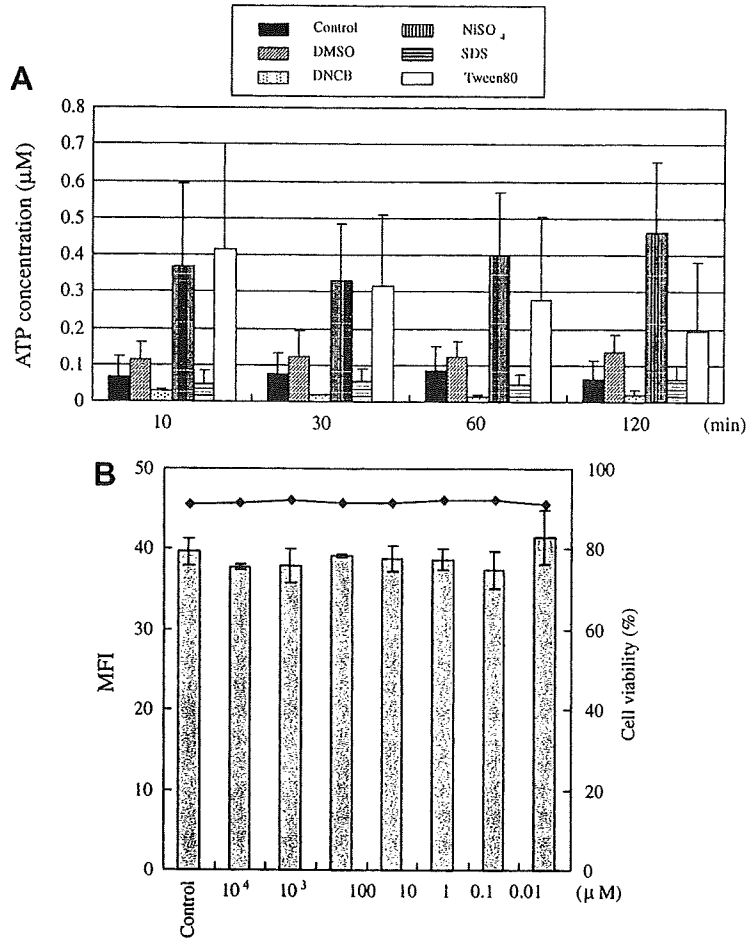

Fig. 4. Effect of ATP on THP-1 cell-surface thiols. (A) Measurement of ATP released from chemical-treated THP-1 cells. The cells were treated with DMSO (2000 μg/mL), DNCB (160 μg/mL), $NiSO_4$ (5000 μg/mL), SDS (50 μg/mL) or Tween80 (5000 μg/mL). After treatment with chemicals for 10, 30, 60 and 120 min, culture medium was collected and the ATP concentration was measured. (B) Effect of ATP on THP-1 cell-surface thiols. Gray bars showed the change of cell-surface thiols and the line chart shows cell viability. Mean values ± SD for at least three independent experiments are shown.

Conflicts of interest statement

None declared.

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Development of an *in vitro* photosensitization assay using human monocyte-derived cells

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ABSTRACT

In this study, with the aim of developing a cell-based *in vitro* photosensitization assay, we examined whether changes of CD86 and CD54 expression on cells of a human monocytic cell line, THP-1, could be used to assess the photosensitizing potential of chemicals. First, we identified suitable conditions of UV-irradiation (irradiation dose; 5.0 J/cm^2, irradiation intensity; 1.7 mW/cm^2) by investigating the effect of UV-irradiation on CD86 and CD54 expression on untreated or 6-methylcoumarin (a representative photoallergen)-treated THP-1 cells (irradiation method). However, acridine, a representative photo-irritant, augmented CD86 and CD54 expression on THP-1 cells, apparently via induction of reactive oxygen species (ROS). In order to abolish the effect of ROS, we examined CD86 and CD54 expression on THP-1 cells treated with pre-irradiated chemicals (pre-irradiation method). We found that UV-irradiated photoallergens, but not photo-irritants, enhanced CD86 and/or CD54 expression on the THP-1 cells. Finally, based on the results of irradiation, non-irradiation, and pre-irradiation with 18 test chemicals, we built a decision tree, which allows us to distinguish between photoallergens and photo-irritants. We suggest that this system may be useful for *in vitro* evaluation of the photoallergic potential of chemicals.

1. Introduction

Evaluation of the safety of ingredients is an important part of the development of cosmetics and drugs. Photosensitization, which includes phototoxicity and photoallergy, is a health hazard arising from exposure of skin to normally harmless levels of light in the presence of a radiation-absorbing material (photosensitizer). Ultraviolet A (UVA) plays a key role (Tokura, 2005). Phototoxicity is the acute toxic response elicited after the first exposure of skin to photosensitizers, followed by irradiation with light. Phototoxicity is mediated by reactive oxygen species (ROS), in particular singlet oxygen (Inbaraj et al., 2005; Onoue and Tsuda, 2005). On the other hand, photoallergy is provoked as a consequence of immunotoxicological response to photosensitizers under light irradiation. Ingredients of cosmetics and drugs that have been reported to induce photocontact dermatitis include antibacterial agents (e.g., 3,3′,4′,5-tetrachlorosalicylanilide), sunscreen agents (e.g., *p*-aminobenzoic acid), fragrances (e.g., 6-methylcoumarin) and non-steroidal anti-inflammatory drugs (e.g., ketoprofen) (Rietchel and Fowler, 2001). Assessments of phototoxicological properties of materials have generally been performed in animal tests employing guinea pigs, rabbits, rats or mice. However, the development of alternative *in vitro* assays is now required following the 7th amendment of the European Cosmetics Directive, published in 2003. For phototoxicity testing, the *in vitro* 3T3 NRU phototoxicity test has been adopted as an alternative to *in vivo* phototoxicity tests (Organization for Economic Cooperation and Development (OECD), 2004). Of course, *in vitro* replacements for the *in vivo* photoallergy test have also been sought. The mechanism of photoallergy involves the conjugation of photoallergens to epidermal cells under UVA irradiation. Subsequently, photoantigen-bearing epidermal Langerhans cells sensitize T cells in draining lymph nodes (Kurita et al., 2007). So far, two models of photoantigen formation, the photohapten model and the prohapten model, have been reported (Tokura et al., 1996; Tokura, 2000). In the induction phase of photoallergy, photoallergens produce ROS and induce apoptosis in keratinocytes, but the use of ROS and apoptosis as biomarkers might result in detection of both photoallergy and phototoxicity (Onoue and Tsuda, 2005; Kurita et al., 2007). Lovell and Jones focused on the formation of photoantigen, and evaluated an *in vitro* test using the photobinding property of photoallergens to human serum albumin (Lovell and Jones, 2000). Barratt et al. reported an *in silico* approach for the prospective identification of photoallergens using a rule-based expert system (Barratt et al., 2000). There have been few reports on *in vitro* cell-based assays able to distinguish photoallergens and photo-irritants.

In our previous reports, we suggested that measuring CD86 and/or CD54 expression on THP-1 (human monocytic leukemia cell

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line) could be used as an *in vitro* skin sensitization assay (Ashikaga et al., 2002, 2006; Sakaguchi et al., 2006). CD86 and CD54 expression on Langerhans cells is increased by exposure to a photoallergen plus UVA irradiation (Nishijima et al., 1999). Therefore, CD86 and/or CD54 expression on THP-1 cells might be useful biomarkers for assessing photoallergic potency. In this study, we evaluated whether induction of CD86 and CD54 expression on THP-1 cells could be used to identify photoallergens and developed a new *in vitro* photosensitization test system based on the results.

2. Materials and methods

2.1. Cells and culture medium

THP-1 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were cultured in RPMI 1640 medium (Invitrogen Corp., Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; SAFC Biosciences, Lenexa, KS, USA), 4 mM L-glutamine (Invitrogen Corp.), and 1% penicillin/streptomycin/fungizone (Invitrogen Corp.).

2.2. Chemicals

Chlorpromazine hydrochloride (CPZ) was from Nacalai Tesque, Inc. (Kyoto, Japan). Benzophenone (2-hydroxy-4-methoxybenzophenone) (BP), *p*-phenylenediamine (PPD), ketoprofen, promethazine hydrochloride (PM), bithionol, *p*-aminobenzoic acid (PABA), piroxicam, enoxacin, chlorhexidine dihydrochloride (CHD), 2,4-dinitrochlorobenzene (DNCB), 5-methoxypsoralen (5-MOP), lactic acid (LA) and sodium lauryl sulfate (SLS) were from Sigma–Aldrich Corp. (St. Louis, MO, USA). 3,3′,4′,5-Tetrachlorosalicylanilide (TCSA) was from Acros Organics N. V. (Geel, Belgium). 6-Methylcoumarin (6-MC) and anthracene were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Acridine was from Lancaster Synthesis, Inc. (Windham, NH, USA). Distilled water (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan), ethanol (Wako Pure Chemical Industries, Ltd.), or RPMI 1640 medium without phenol red (Invitrogen Corp.) (medium (−)) was selected as a solvent. FITC-labeled anti-CD86 antibody was purchased from BD Biosciences Pharmingen (Clone: Fun-1, San Diego, CA, USA). FITC-labeled anti-CD54 and anti-mouse IgG1 was purchased from DAKO (Glostrup, Denmark). Gammaglobulins from human blood were from Sigma–Aldrich Corp.

2.3. Irradiation

A SOL 500 Sun Simulator (Dr. Hönle AG, Gräfelfing, Germany) equipped with a 500 W metal halide lamp and a H1 filter to attenuate UVB was used or irradiation. A UVA-Meter (Dr. Hönle AG) was used to measure UVA intensity.

2.4. Test procedure

2.4.1. Irradiation and non-irradiation methods

For treatment, the test chemical was dissolved in distilled water, ethanol, or medium (−), diluted with medium (−), and dispensed into a 24-well plate. The final concentration of each solvent was 0.5%. THP-1 cells were washed with medium (−) and seeded in the aforementioned plate (1×10^6 cells/mL; 1 mL/well). Plates were irradiated (irradiation method) or keep in the dark (non-irradiation method).

2.4.2. Pre-irradiation method

In the pre-irradiation method, the irradiation was applied to a 24-well plate into which medium (−) had been dispensed and test chemical added. After incubation for 30 min at 37 °C, THP-1 cells suspended in medium (−) were seeded into the wells (1×10^6 cells/mL; 1 mL/well).

2.4.3. Flow cytometric analysis

After treatment for 24 h with chemicals under the conditions as described in Sections 2.4.1 and 2.4.2, the cells were washed twice with PBS containing 0.1% BSA, then treated with 0.01% globulins, Cohn fraction II, III (Sigma–Aldrich), for 10 min on ice to block FcR. The cells were then washed once with PBS containing 0.1% BSA. Staining was performed with FITC-conjugated anti-human CD86 antibody, anti-human CD54 antibody or IgG1 at 4 °C for 30 min. The expression of CD86 and CD54 on viable cells unstained with 0.5% propidium iodide solution (Sigma–Aldrich Corp.) was measured with a flow cytometer (EPICS XL/XL-MCL System II, Beckman Coulter, Inc., Harbor Boulevard, CA, USA). In total, 10,000 living cells were analyzed. In principle, cells were treated with test chemical at a concentration giving around 75% cell viability under the conditions used.

2.5. Calculation of CD86 and CD54 expression

RFI of CD86 and CD54 expression was used as an indicator of activation of THP-1 cells. RFI was calculated by use of the following formula: CD86 or CD54 expression (% of control) = 100 × (mean fluorescence intensity (MFI) of sample-treated or UV-irradiated

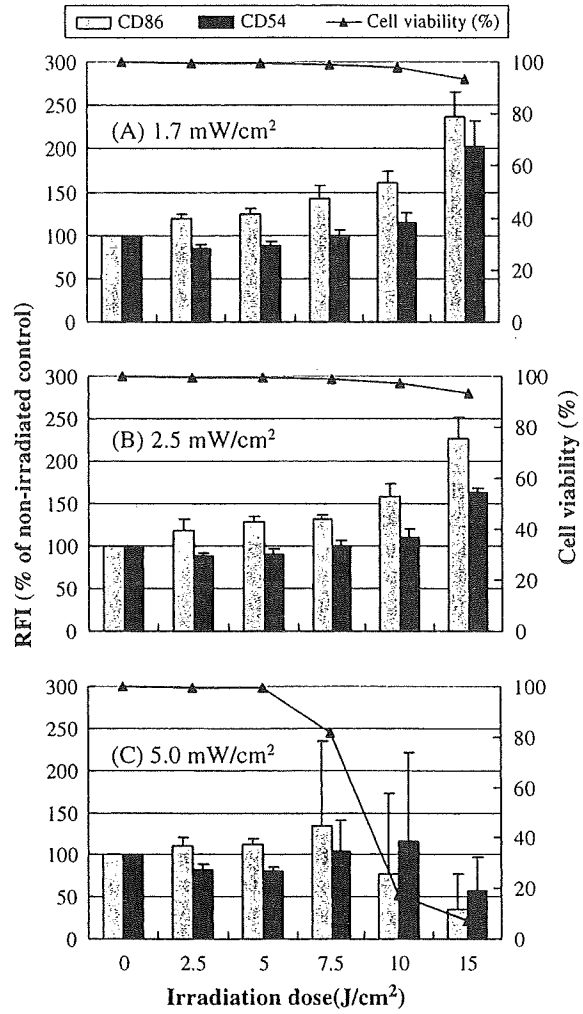

Fig. 1. Effect of intensity and total amount of irradiation on CD86 and CD54 expression and viability of na THP-1 cells. Cells were irradiated at the doses of (A) 1.7 mW/cm², (B) 2.5 mW/cm² and (C) 5 mW/cm². After irradiation, THP-1 cells were incubated for 24 h at 37 °C. The expression of CD86 and CD54 and the cell viability were measured by flow cytometry and RFIs (% of non-irradiated control) were calculated. Mean values ± S.D. for at least three independent experiments are shown.

cells (anti-CD86 or CD54 antibody treated) – MFI of sample-treated or UV-irradiated isotype control cells (IgG1 treated))/(MFI of vehicle-treated or non-irradiated cells (anti-CD86 or CD54 antibody treated) – MFI of vehicle-treated or non-irradiated isotype control cells (IgG1 treated)).

2.6. Data analysis

Tests were performed at least three times on each chemical and the values of CD86 and CD54 expression and cell viability were calculated, each as the mean of at least three test results. If the cell viability was less than 50%, the data for the corresponding concentration of the test chemical were not used for the judgment.

3. Results

3.1. Effects of irradiation

We examined the effect of intensity and total amount of irradiation on THP-1 cells. Irradiation caused a dose-related increase of CD86 and CD54 expression on THP-1 cells, especially at the intensities of 1.7 and 2.5 mW/cm^2 (Fig. 1A and B). At the intensity of 5.0 mW/cm^2, cytotoxicity emerged around the irradiation dose of 7.5 J/cm^2 (Fig. 1C).

3.2. Effects of irradiation in the presence of a photoallergen

To ascertain the effect of irradiating THP-1 cells in the presence of a photoallergen, cells were treated with 6-methylcoumarin (6-MC), a representative photoallergen, under various conditions of irradiation. The maximum value of CD86 expression on THP-1 cells was little affected by irradiation (Fig. 2). However, CD54 expression was upregulated by irradiation and reached a maximum at the irradiation dose of 5.0 J/cm^2 at the intensity of 1.7 mW/cm^2. The cytotoxic effect of 6-methylcoumarin was dependent on the irradiation dose. The irradiation dose of 5.0 J/cm^2 at the intensity of 1.7 mW/cm^2 was chosen as an appropriate test condition to detect the effects of photoallergens on THP-1 cells.

3.3. Effects of irradiation in the presence of a photoirritant

The effects of a phototoxic material on THP-1 cells were confirmed under the optimized irradiation conditions. Acridine, a photo-irritant, increased the expression of CD86 and CD54 on THP-1 cells under irradiation (Fig. 3). To determine the mechanism of activation of THP-1 cells by phototoxic chemicals, the effect of hydrogen peroxide, a representative reactive oxygen species (ROS), was examined. Expression of both CD86 and CD54 was dose-dependently increased in THP-1 cells exposed to hydrogen peroxide (Fig. 4).

3.4. Effects of a pre-irradiated photoallergen or a pre-irradiated photoirritant

In order to eliminate the effect of ROS on CD86 and CD54 expression, we used the pre-irradiation method to investigate changes of CD86 and CD54 expression on acridine-treated THP-1 cells. Compared with the irradiation method, the extent of up-regulation was reduced when the pre-irradiation method was used (Fig. 5A). On the other hand, 6-MC, a representative photoallergen, induced CD86 expression to the extent of over 150% of RFI, which is the tentatively proposed cut-off value (Sakaguchi et al., 2006), in the pre-irradiation method (Fig. 5B).

3.5. Effects of a panel of test chemicals using non-irradiation, irradiation and pre-irradiation methods

To further evaluate the pre-irradiation method, we evaluated more chemicals using the non-irradiation, irradiation and pre-irradiation methods. Eighteen test chemicals were selected (LeVine, 1984; Hariya et al., 1993; Tokura, 1998; National Toxicology Program, 1999; Nishijima et al., 1999; Lovell and Jones, 2000; Landers et al., 2003) and evaluated (Table 1). Dose-setting was determined based on the CV_{75} value (estimated dose of 75% cell viability) as a sub-toxic concentration, as described by Sakaguchi et al. (2009). Three arbitrary thresholds were selected: RFI values of 150%, 200% and 250%. The minimum concentrations of test chemicals which were judged as positive in terms of changes in CD86 or CD54

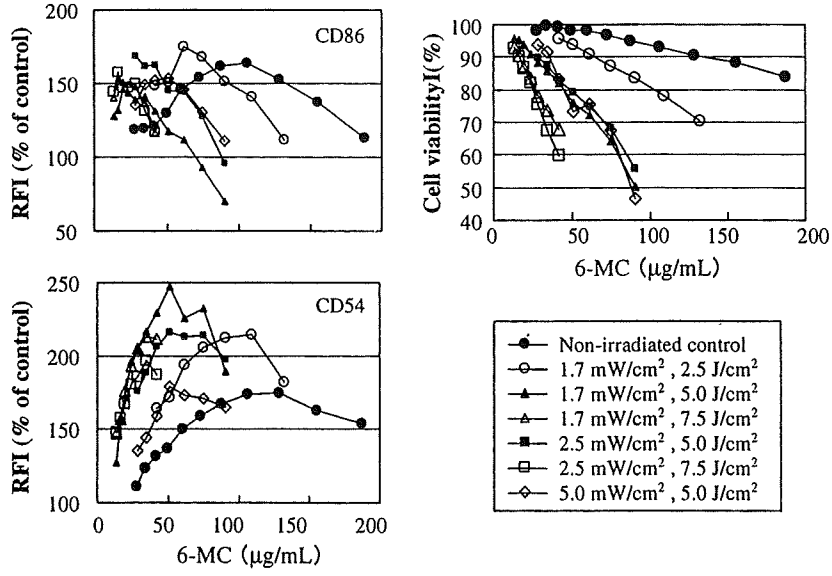

Fig. 2. Effect of intensity and total amount of irradiation on CD86 and CD54 expression and viability of 6-MC-treated THP-1 cells. Cells were irradiated at various intensities and total amounts of irradiation as shown, after treatment with various concentrations of 6-MC. After irradiation, cells were incubated for 24 h at 37 °C. The expression of CD86 and CD54 and the cell viability were measured by flow cytometry and RFIs were calculated. Mean values ± S.D. for at least three independent experiments are shown.