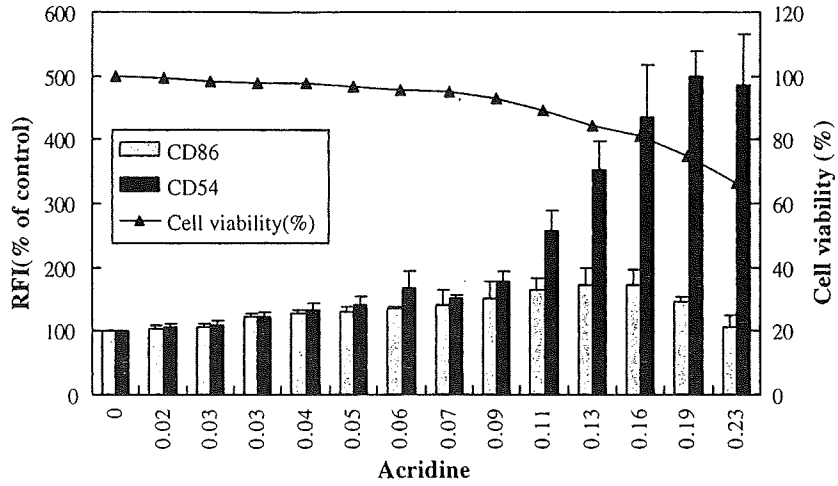

Fig. 3. Activation of THP-1 cells by acridine under irradiation. Cells were treated with various concentrations of acridine, and after irradiation, were incubated for 24 h at 37 °C. The expression of CD86 and CD54 and the cell viability were measured by flow cytometry and RFIs were calculated. Mean values ± S.D. for at least three independent experiments are shown.

expression at each threshold were designated as EC150, EC200 and EC250, and the values obtained are summarized in Table 2.

3.6. Development of a decision tree for general cytotoxicity, phototoxicity, allergy and photoallergy

To identify photoallergens, photo-irritants, allergens and non-sensitizers using the non-irradiation, irradiation and pre-irradiation methods, we establish a tiered evaluation system to predict the photoallergic and phototoxic potential of substances (Fig. 6). Step 1: when the expression of both CD86 and CD54 in the non-irradiation and irradiation methods was judged as negative, the photoallergic, allergic and phototoxic potentials of chemical are considered to be negative. Step 2: when the ratio of EC200 in the non-irradiation method to that in the irradiation method (EC200 (non-irradiation)/EC200 (irradiation) value) is below three or when the RFI value of CD86 or CD54 expression in the irradiation method is below 200% at all set doses, the photoallergic and phototoxic potentials of the chemical are considered to be negative but the allergic potential is considered to be positive. If the EC200 value of CD86 is different from that of CD54, the lower concentration is selected for calculation. Chemicals whose EC200 (non-irradiation)/EC200 (irradiation) value is over 3 are further tested using the pre-irradiation method. Step 3: when RFI of 150% (CD86 expression) or RFI of 250% (CD54 expression) is applied as the tentative criterion in the pre-irradiation method, chemicals which are judged as negative are considered to be phototoxic. Step 4: when chemicals judged as positive in the pre-irradiation method are judged as negative in the non-irradiation method, their photoallergic (not allergic) potential is considered to be positive. On the other hand, when a chemical is judged as positive in both the pre-irradiation and non-irradiation method, it is considered to show both photoallergic and allergic potential.

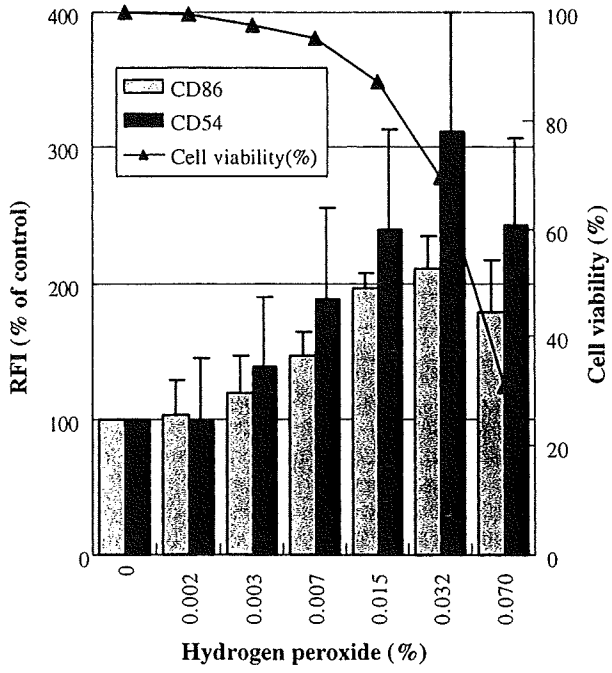

Fig. 4. Effect of hydrogen peroxide on THP-1 cells. Cells were treated with various concentrations of hydrogen peroxide for 24 h at 37 °C. The expression of CD86 and CD54 and the cell viability were measured by flow cytometry and RFIs were calculated. Mean values ± S.D. for at least three independent experiments are shown.

The result of evaluation of 18 chemicals are summarized in Table 3. In Step 1, among 18 chemicals, SDS, LA, PABA and piroxicam were judged as negative. In Step 2, EC200 (non-irradiation)/EC200 (irradiation) values of TCSA, CHD and DNCB were below three. These chemicals were judged as allergenic. In Step 3, CD86 or CD54 expression of enoxacin, 5-MOP and acridine was not augmented in the pre-irradiation method. These chemicals were judged as phototoxic. In Step 4, CD86 or CD54 expression of 6-MC, bithionol and anthracene was not augmented in the non-irradiation method. These chemicals were judged as photoallergenic. On the other hand, CPZ, BP, PPD, ketoprofen and PM, which were judged as positive in all steps, were evaluated as photoallergenic/allergenic. Among 18 chemicals, 13 chemicals were judged correctly, compared with *in vivo* data (LeVine, 1984; Hariya et al., 1993; Tokura, 1998; National Toxicology Program, 1999; Nishijima et al., 1999; Lovell and Jones, 2000; Landers et al., 2003). Among five chemicals which were misjudged, TCSA (*in vivo*; photoallergenic, phototoxic and allergenic) was judged as allergenic, enoxacin (*in vivo*; photoallergenic), was judged as phototoxic, anthracene (*in vivo*; phototoxic), was judged as photoallergenic, and PABA and piroxicam (*in vivo*; photoallergenic) were judged as negative.

4. Discussion

A flow cytometric method using human monocytic leukemia cell line, THP-1, has been developed and validated as an *in vitro*

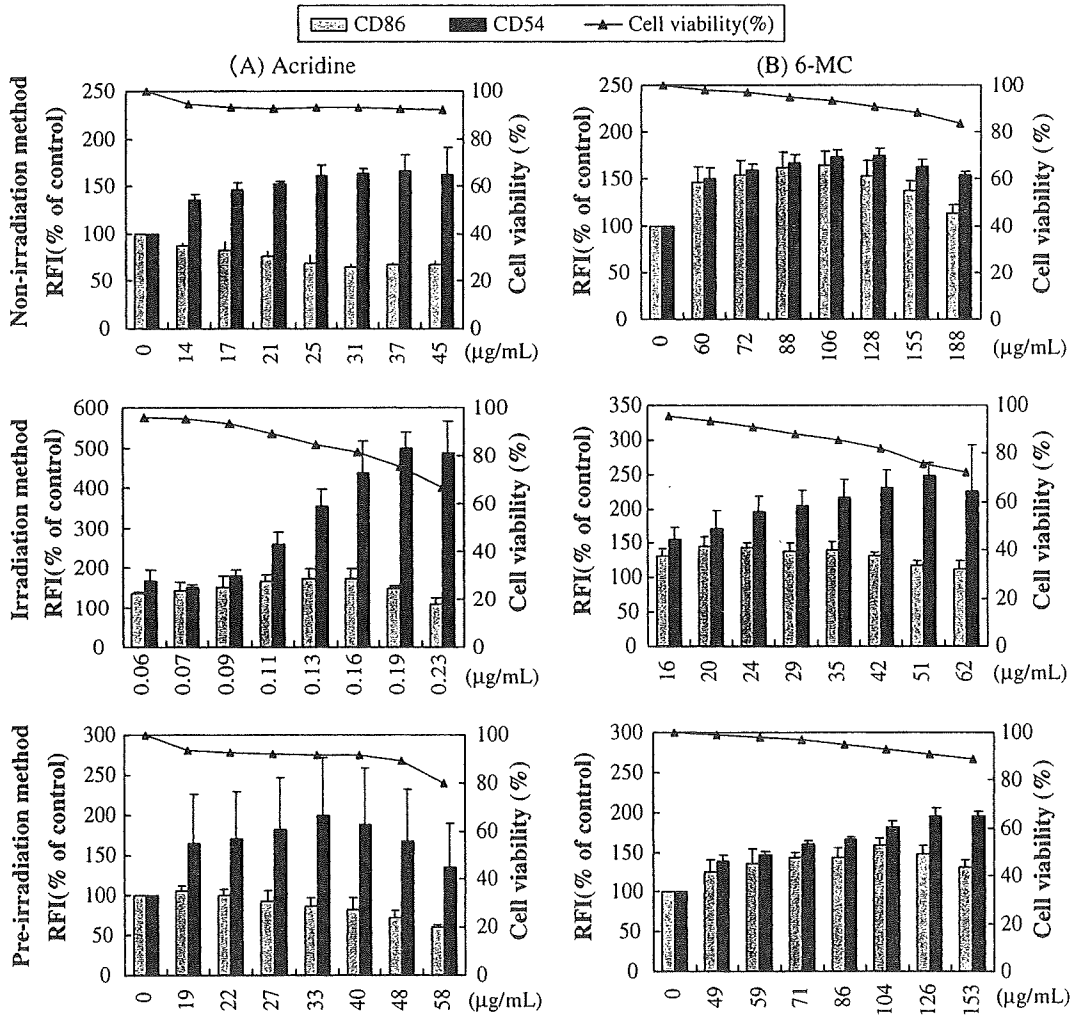

Fig. 5. Activation of THP-1 cells by acridine (a photo-irritant) and 6-MC (a photo-allergen) determined with the non-irradiation, irradiation and pre-irradiation methods. Cells were treated with various concentrations of acridine or 6-MC, then irradiated according to the irradiation and pre-irradiation methods described in Section 2, and incubated for 24 h at 37 °C. In the non-irradiation method, cells were incubated for 24 h at 37 °C without irradiation after treatment with acridine or 6-MC. 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.

Table 1
CV_{75} value of test chemicals.

Test samples		CV75 (μg/mL)			
		Non-irradiation method	Irradiation method	Pre-irradiation method	Solvent
CPZ	Chlorpromazine HCl	7.3	0.9	29	Saline
BP	Benzophenone	104	13	123	Ethanol
TCSA	3,3′,4′,5-Tetrachlorosalicylanilide	2.6	15	N.D.	Ethanol
PPD	*p*-Phenylenediamine	10	1.3	8.2	Saline
Ketoprofen	–	528	17	110	Ethanol
PM	Promethazine HCl	26	1.6	38	Ethanol
6-MC	6-Methylcoumarin	188	62	153	Ethanol
Bithionol	–	104	13	123	Ethanol
PABA	*p*-Aminobenzoic acid	2500	2066	2500	Ethanol
Piroxicam	–	100	100	N.D.	Ethanol
Enoxacin	–	100	28	100	Ethanol
CHD	Chlorhexidine HCl	23	8.3	N.D.	Ethanol
DNCB	2,4-Dinitrochlorobenzene	5.0	0.6	3.7	Ethanol
Anthracene	–	50	50	50	Ethanol
5-MOP	5-Methoxypsoralen	50	8.9	50	Ethanol
Acridine	–	45	0.2	58	Ethanol
SLS	Sodium lauryl sulfate	85	70	N.D.	Saline
LA	Lactic acid	2801	2844	N.D.	Medium

sensitization test (Ashikaga et al., 2002, 2006; Sakaguchi et al., 2006). THP-1 is used as a substitute for dendritic cells, which are involved in skin allergic reaction, for the study of alternatives to animal experiments. Several studies have confirmed the usefulness

Table 2
Summary data for changes of CD86 and CD54 expression on THP-1 cells exposed to 18 chemicals according to the non-irradiation, irradiation and pre-irradiation methods. Relative fluorescence intensities (RFIs) of CD86 and CD54 on THP-1 were compared using three tentative criteria in the non-irradiation, irradiation and pre-irradiation methods. The minimum concentrations which were judged as positive for CD86 or CD54 expression under each threshold are indicated as EC150, EC200 and EC250. "N.D." means that EC150, EC200 or EC250 was not determined because CD86 or CD54 expression did not exceed the relevant criterion. "N.T." means that CD86 and CD54 expression was not tested in the pre-irradiation method.

Test samples	Non-irradiation method						Irradiation method						Pre-irradiation method					
	CD86			CD54			CD86			CD54			CD86			CD54		
	EC150 (μg/mL)	EC200 (μg/mL)	EC250 (μg/mL)	EC150 (μg/mL)	EC200 (μg/mL)	EC250 (μg/mL)	EC150 (μg/mL)	EC200 (μg/mL)	EC250 (μg/mL)	EC150 (μg/mL)	EC200 (μg/mL)	EC250 (μg/mL)	EC150 (μg/mL)	EC200 (μg/mL)	EC250 (μg/mL)	EC150 (μg/mL)	EC200 (μg/mL)	EC250 (μg/mL)
CPZ	3.1	7.2	N.D.	4.3	5.6	6.3	0.3	N.D.	N.D.	0.2	0.3	0.3	19	24	N.D.	12	17	18
BP	42	68	N.D.	38	67	N.D.	8.7	N.D.	N.D.	4.8	6.9	9.8	44	83	N.D.	<39	<39	48
TCSA	N.D.	N.D.	N.D.	<0.8	1.2	N.D.	N.D.	N.D.	N.D.	4.8	7.6	9.8	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.
PPD	2.1	4.4	N.D.	6.9	N.D.	N.D.	0.9	N.D.	N.D.	0.8	1.0	1.2	4.0	7.2	N.D.	<3.8	6.0	8.1
Ketoprofen	N.D.	N.D.	N.D.	172	193	311	7.1	N.D.	N.D.	<5.4	6.0	6.8	<35	N.D.	N.D.	<35	37	60
PM	N.D.	N.D.	N.D.	8.4	10	12	1.0	N.D.	N.D.	<0.7	1.0	1.0	N.D.	N.D.	N.D.	12	15	18
6-MC	66	N.D.	N.D.	60	N.D.	N.D.	N.D.	N.D.	N.D.	16	26	N.D.	94	N.D.	N.D.	62	N.D.	N.D.
Bithionol	N.D.	N.D.	N.D.	<7.1	N.D.	N.D.	N.D.	N.D.	N.D.	11	14	17	N.D.	N.D.	N.D.	<27	43	45
PABA	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	697	N.D.	N.D.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.
Piroxicam	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.
Enoxacin	N.D.	N.D.	N.D.	65	N.D.	N.D.	<9.5	N.D.	N.D.	<9.5	21	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
CHD	N.D.	N.D.	N.D.	<4.2	18	N.D.	N.D.	N.D.	N.D.	<2.6	N.D.	N.D.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.
DNCB	<0.67	1.2	1.4	0.7	1.3	2.2	0.2	0.5	N.D.	0.4	N.D.	N.D.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.
Anthracene	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	20	36	N.D.	20	28	35	<16	N.D.	N.D.	<16	<16	16
5-MOP	19	N.D.	N.D.	20	22	N.D.	1.4	2.8	N.D.	4.1	N.D.	N.D.	N.D.	N.D.	N.D.	24	N.D.	N.D.
Acridine	N.D.	N.D.	N.D.	19	N.D.	N.D.	0.09	N.D.	N.D.	0.05	0.10	0.11	N.D.	N.D.	N.D.	<19	33	N.D.
SLS	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.
LA	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.

Table 3
Relationship between *in vivo* data and the results of the present *in vitro* photosensitization test based on the proposed decision tree. *In vivo* data were taken from the literature (LeVine, 1984; Hariya et al., 1993; Tokura, 1998; National Toxicology Program, 1999; Nishijima et al., 1999; Lovell and Jones, 2000; Landers et al., 2003). "(a)/(b)" means the ratio of EC200 value in the non-irradiation method (a) to that in the irradiation method (b), and was calculated for each of CD86 and CD54. "N.D." means that EC200 was not determined because CD86 or CD54 expression did not exceed the criterion (RFI > 200%). "N.C." means that "(a)/(b)" could not be calculated because the EC200 value in the irradiation method could not be determined. ">3" was assigned when CD86 or CD54 expression was above the criterion level in the irradiation method, but not the non-irradiation method. "N.T." means that CD86 and CD54 expression was not tested in the pre-irradiation method. +: positive, −: negative.

Test samples	*In vivo* data			*In vitro* test data									
	Photo-allergenicity	Photo-toxicity	Allergenicity	Non-irradiation method (a) EC200 value (mg/mL)		Irradiation method (b)		(a)/(b)		Pre-irradiation method		Judgment	References
				CD86	CD54	CD86	CD54	CD86	CD54	CD86	CD54		
CPZ	+	+	+	7.2	5.6	N.D.	0.3	N.C.	19	+	+	Photoallergic/allergic	Lovell and Jones (2000)
BP	+	−	+	68	67	N.D.	6.9	N.C.	10	+	+	Photoallergic/allergic	Landers et al. (2003)
TCSA	+		+	N.D.	1.2	N.D.	7.6	N.C.	0.15	N.T.	N.T.	Allergic	Nishijima et al. (1999), National Toxicology Program (1999)
PPD	+		+	4.4	N.D.	N.D.	1.0	N.C.	>3	+	+	Photoallergic/allergic	LeVine (1984), National Toxicology Program (1999)
Ketoprofen	+	−	+	N.D.	193	N.D.	6.0	N.C.	32	+	+	Photoallergic/allergic	Lovell and Jones (2000)
PM	+	+	+	N.D.	10	N.D.	1.0	N.C.	11	−	+	Photoallergic/allergic	Lovell and Jones (2000)
6-MC	+	−	−	N.D.	N.D.	N.D.	26	N.C.	>3	+	−	Photoallergic	Lovell and Jones (2000)
Bithionol	+	+		N.D.	N.D.	N.D.	14	N.C.	>3	−	+	Photoallergic	Lovell and Jones (2000)
PABA	+	−	−	N.D.	N.D.	N.D.	N.D.	N.C.	N.C.	N.T.	N.T.	Negative	Lovell and Jones (2000)
Piroxicam	+			N.D.	N.D.	N.D.	N.D.	N.C.	N.C.	N.T.	N.T.	Negative	Hariya et al. (1993)
Enoxacin	+			N.D.	N.D.	N.D.	21	N.C.	>3	−	−	Phototoxic	Tokura (1998)
CHD	−	−	+	N.D.	18	N.D.	N.D.	N.C.	N.C.	N.T.	N.T.	Allergic	Lovell and Jones (2000)
DNCB	−	−	+	1.2	1.3	0.5	N.D.	2.7	N.C.	N.T.	N.T.	Allergic	
Anthracene	−	+	−	N.D.	N.D.	36	28	>3	>3	+	+	Photoallergic	Lovell and Jones (2000)
5-MOP	−	+	+	N.D.	22	2.8	N.D.	>3	>3	−	−	Phototoxic	Lovell and Jones (2000)
Acridine	−	+	−	N.D.	N.D.	N.D.	0.10	N.C.	>3	−	−	Phototoxic	Lovell and Jones (2000)
SLS	−	−	−	N.D.	N.D.	N.D.	N.D.	N.C.	N.C.	N.T.	N.T.	Negative	Lovell and Jones (2000)
LA	−	−	−	N.D.	N.D.	N.D.	N.D.	N.C.	N.C.	N.T.	N.T.	Negative	

of this cell line for *in vitro* sensitization tests. Here, we confirmed that it can be used to assess photoallergenicity, as well as allergenicity.

We found that the expression of CD86 and CD54 on THP-1 cells under irradiation was up-regulated dose-dependently. At high irradiation intensity, cytotoxicity was observed. Ultraviolet light irradiation *in vivo* is known to induce immunosuppression, and this is attributed to the sensitivity of several types of cells in the skin to ultraviolet light. The immunosuppression involves perturbed expression of several co-stimulatory molecules, including CD86 and CD54 on dendritic cells (Denfeld et al., 1998; Rattis et al., 1998; Dittmar et al., 1999; Iwai et al., 1999; Nakagawa et al., 1999,

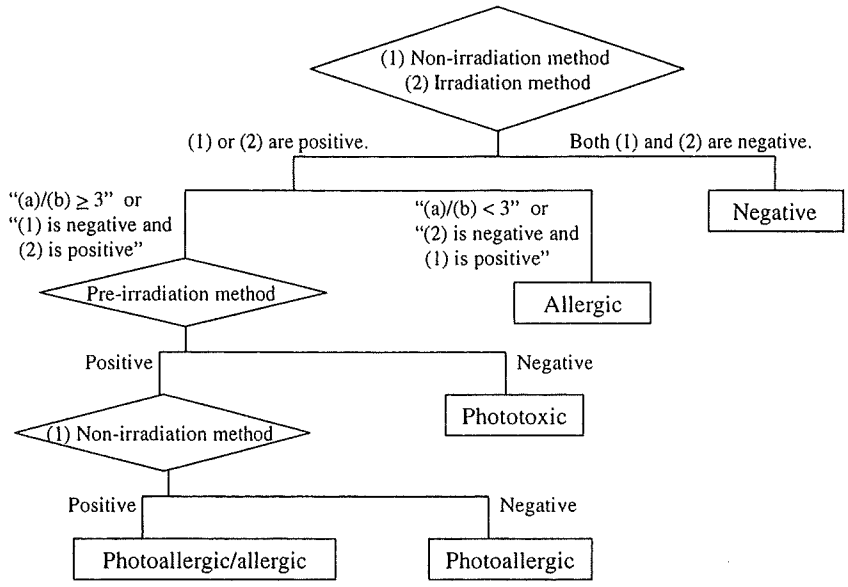

Fig. 6. Proposed decision tree for predicting photoallergenicity, phototoxicity, and allergenicity. If the RFI of either CD86 or CD54 exceeds the criterion, the chemical is positive. If the RFIs of CD86 and CD54 are below the criterion, the chemical is negative. In the (1) non-irradiation and (2) irradiation methods, RFI of 200% (CD86 and CD54 expression) was applied as a tentative criterion. In the pre-irradiation method, RFI of 150% (CD86 expression) or RFI of 250% (CD54 expression) was applied. "(a)/(b)" indicated the ratio of EC200 in the non-irradiation method (a) to that in the irradiation method (b).

2004; Laihia and Jansen, 2000; Mittelbrunn et al., 2005). Ultraviolet light also induced apoptosis of dendritic cells (Rattis et al., 1998; Aubin, 2003; Nakagawa et al., 2004; Timares et al., 2008). These facts imply that at least some of the photodynamic mechanisms that occur *in vivo* are replicated under the test conditions *in vitro*. Suitable irradiation conditions for the test (1.7 mW/cm^2, 5 J/cm^2), which minimized cytotoxicity, were determined using 6-MC as a representative photoallergen. The irradiation conditions are identical to those of the 3T3 NRU PT, for which the same irradiation apparatus is used. Interestingly, a fixed intensity of irradiation, 5 J/cm^2, has been indicated to be necessary and sufficient for eliciting photocontact allergic reactions in the photopatch test (Hasan and Jansen, 1996).

Under the test conditions used for the irradiation method, photo-irritants, as well as photoallergens, induced CD86 and CD54 expression on THP-1 cells. ROS including hydrogen peroxide, which is generated by UV-activated photo-irritants, appear to be involved (Fig. 4). Indeed, dendritic cells are activated by ROS, especially hydrogen peroxide (Caceres-Dittmar et al., 1995; Rutault et al., 1999). To discriminate photoallergens from photo-irritants, we examined the pre-irradiation method, in which THP-1 cells were treated with irradiated test chemicals, in addition to the non-irradiation and irradiation methods. Hino et al. reported a similar approach to examine the prohaptenic ability of chemicals (Hino et al., 2008). The augmentation of CD86 expression by 6-MC, but not acridine, in the pre-irradiation method might be explained by photo-induced binding of test substance or photodecomposition products, but not short-lived ROS, to THP-1 cells. CD86 and/or CD54 expression on THP-1 cells is thus upregulated by photoallergens, but not photo-irritants.

Based on the results of the non-irradiation, irradiation and pre-irradiation methods using 18 chemicals, we prepared a decision tree to distinguish between photoallergens and photo-irritants (Fig. 6). The photoallergenicity of seven out of 11 photoallergic test chemicals was correctly assessed by using CD86 and CD54 as biomarkers. It has already been reported that the extents of increase of CD86 and CD54 expression are not necessarily the same (Sakaguchi et al., 2006). Furthermore, our data indicated that there were some chemicals that induced either CD86 or CD54 expression in the non-irradiation, irradiation and pre-irradiation methods. Thus, CD86 and CD54 were considered to be complementary biomarkers. This decision tree appears to be suitable in part for identifying photoallergens. However, the *in vitro* 3T3 NRU phototoxicity test (OECD, 2004) seems to be more suitable for detecting phototoxic potential only, because the phototoxicity of chemicals which were judged in that test as "photoallergenic and allergenic" or "photoallergenic" such as CPZ, PM and bithionol was not detected using our decision tree. So, a battery system combining this decision tree and the *in vitro* 3T3 NRU phototoxicity test is an interesting possibility. In our decision tree, TCSA, which is photoallergenic and allergenic *in vivo*, was only assessed as allergenic. TCSA is known to bind noncovalently to one major site on human serum albumin and to form characteristic photoconjugates. The special role of albumin as a carrier protein in TCSA photoallergy has been reported (Barratt et al., 1987; Pendlington and Barratt, 1990). Thus, it may be important to consider the role of carriers in the pre-irradiation method. In addition, PABA and piroxicam were classified as negative. Both showed low cytotoxicity towards THP-1 cells under the test conditions. Cytotoxicity of test chemicals towards THP-1 cells is required for detecting sensitizing potential in *in vitro* sensitization tests (Yoshida et al., 2003). The cytotoxic effect of these chemicals on Langerhans cells should be verified.

In conclusion, we have developed a new *in vitro* photosensitization using a cultured cell line, THP-1, and confirmed its utility to predict the photoallergic potential of various chemicals. A small number of individuals who develop photoallergic contact dermatitis following exposure to photosensitizing chemicals retain a persistent reactivity to light after all exposure to the chemicals has ceased (persistent light reaction; PLR) (Rietchel and Fowler, 2001). Thus, identification of chemicals with photosensitizing potential may reduce the risk of PLR, as well as being important for immunology and dermatoxicology research. Further development of this method, perhaps as part of a battery system, is expected to provide a useful tool for predicting photoallergenicity.

Acknowledgment

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Original Article

Modification of cell-surface thiols elicits activation of human monocytic cell line THP-1: Possible involvement in effect of haptens 2,4-dinitrochlorobenzene and nickel sulfate

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ABSTRACT — Human monocytic cell line THP-1 cells are used as an indicator for *in vitro* skin sensitization testing. Although p38 mitogen-activated protein kinases (MAPKs) and intracellular redox imbalance play crucial roles in the activation of THP-1 by skin sensitizers, the trigger of cell activation has not been identified. Therefore, we examined whether haptens induce THP-1 maturation directly or indirectly. 2,4-Dinitrochlorobenzene (DNCB), but not dinitrophenol (DNP)-conjugated bovine serum albumin or DNP-conjugated fetal bovine serum, induced CD86 expression. DNCB and nickel sulfate ($NiSO_4$) also induced related changes of cell-surface thiols and phosphorylation of p38 MAPK. However, DNCB is membrane-permeable, and so its direct effect may not be confined to cell membrane proteins. Next, we found that CD86 expression and macrophage inflammatory protein-1β (MIP-1β) production were augmented by the membrane-impermeable thiol blocker 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), and these changes were suppressed by an inhibitor of the p38 MAPK pathway, SB203580. Finally, we confirmed that endocytotic activity for bovine serum albumin (BSA) Alexa Fluor 488 conjugate did not affect cell-surface thiols on THP-1 cells. Thus, our data indicate that the changes of cell-surface thiols are one of the triggers of maturation, and play a key role in activation of THP-1 cells by haptens.

Key words: Cell-surface thiols, Cell activation, p38 MAPK, CD86, MIP-1β, THP-1

INTRODUCTION

Several groups have recently developed *in vitro* assays to predict sensitizing potential of chemicals. These approaches were based on measuring expressions of an appropriate biomarker(s) of an indicator cell line. Dendritic cells, including Langerhans cells, are considered as good candidates for indicator cells, because they are potent antigen-presenting cells and play an important role in induction of skin sensitization by simple chemicals (Aiba and Tagami, 1998). It was demonstrated *in vitro* that human monocyte-derived dendritic cells respond to haptens, such as nickel chloride ($NiCl_2$) and 2,4-dinitrochlorobenzene (DNCB), but not to irritants, such as sodium dodecyl sulfate (SDS), by significantly augmenting their expression of CD86, CD54 and HLA-DR (Aiba *et al.*, 1997; Coutant *et al.*, 1999). Furthermore, *in vitro* sensitization methods using cell lines such as THP-1 (monocytic leukemia cell line) (Ashikaga *et al.*, 2006; Yoshida *et al.*, 2003; Sakaguchi *et al.*, 2006), KG-1 (acute myelogenous leukemia cell line) (Hulette *et al.*, 2001; Yoshida *et al.*, 2003), U-937 (histiocytic lymphoma cell line) (Sakaguchi *et al.*, 2006) and MUTZ-3 (Azam *et al.*, 2006) have also been reported. In these methods, expression of surface biomarkers, such as HLA-DR, CD54 and CD86, is measured by flow cytometry after treatment of cells with haptens, such as DNCB and *p*PD. The THP-1 cell line was reported to be a good candidate indicator for *in*

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vitro sensitization methods from the viewpoint of inter-laboratory reproducibility (Sakaguchi *et al.*, 2006).

p38 mitogen-activated protein kinase (MAPK) plays a crucial rule in the augmentation of CD86 expression on dendritic cells, and intracellular redox imbalance is a very important upstream signal for p38 MAPK (Aiba *et al.*, 2003; Mizuashi *et al.*, 2005). Furthermore, it was reported that intracellular redox imbalance and redox-related expression of genes such as heme oxygenase-1 (HO-1) gene upon treatment with haptens were observed in THP-1 cells (Mizuashi *et al.*, 2005; Hirota and Moro, 2006). Filomeni *et al.* (2003) reported that oxidation of exofacial membrane thiol groups by exogenous membrane-impermeable oxidative-form glutathione (GSSG) triggered a decrease of intracellular glutathione (GSH) content, phosphorylation of p38 MAPK and apoptosis in U-937 cells. Furthermore, Becker *et al.* (2003) reported that coupling of sensitizers to thiol groups is a key event for activation of monocytes and monocyte-derived dendritic cells. These observations might indicate that haptens activated p38 MAPK through redox signals arising from oxidation of cell-membrane thiols. However, skin sensitizers generally have protein- and peptide-binding activity, or acquire activity by metabolic activation, so that hapten-protein or hapten-peptide complexes incorporated into antigen-presenting cells are presented to T cells in the induction phase of skin sensitization. Becker *et al.* (1992) and Girolomoni *et al.* (1990) reported that receptor-mediated endocytosis by Langerhans cells is an early event in the induction phase of skin sensitization. Thus, it remains to establish whether antigen-presenting cells and cell lines are activated by hapten-conjugated protein or by haptens directly. In this paper, we focus on a trigger of THP-1 maturation, and our results indicate that direct change of cell-surface thiols by hapten is more important in activation of THP-1 cells.

MATERIALS AND METHODS

Chemicals

DNCB, nickel sulfate hexahydrate ($NiSO_4$) and SDS were purchased from Sigma-Aldrich Corporation (St. Louis, MI, USA). 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) was purchased from Dojindo Molecular Technologies (Gaithersburg, MD, USA). Bovine serum albumin (BSA) was purchased from Wako Pure Chemicals (Osaka, Japan). Dimethyl sulfoxide (DMSO) was purchased from Kanto Chemical (Tokyo, Japan). Nonpermeable thiol reactive compound Alexa Fluor 488 C_5 maleimide, Alexa Fluor 568 C_5 maleimide, nonpermeable amine reactive compound Alexa Fluor 488 carboxylic acid succinimidyl ester, BSA Alexa Fluor 488 conjugate were purchased from Invitrogen life technologies (Carlsbad, CA, USA). FITC-labeled anti-CD86 antibody was purchased from BD-PharMingen (Clone: Fun-1, San Diego, CA, USA). FITC-labeled anti-mouse IgG1 was purchased from DAKO (Glostrup, Denmark). SB203580, an inhibitor of the p38 MAPK pathway, was purchased from Calbiochem (La Jolla, CA, USA).

Cells and culture

THP-1 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). These cells were maintained in RPMI 1640 medium (Invitrogen life technologies) with 1 % (v/v) antibiotic-antimycotic (Invitrogen life technologies), 10 % fetal bovine serum (v/v) (FBS, JRH Biosciences, Lenexa, KS, USA) and 0.05 mM 2-mercaptoethanol (2-ME) (Invitrogen life technologies) at 37°C in a 5 % CO_2 incubator. Cells were passaged by addition of fresh medium twice a week and cell density was maintained between 0.1 and 0.5 x 10^6 cells/ml.

Preparation of dinitrophenol (DNP)-conjugated BSA and DNP-conjugated FBS

100 mg/ml DNCB was dissolved in DMSO and added to 10 mg/ml BSA solution or RPMI 1640 medium containing 10 % FBS. The proteins in RPMI 1640 medium containing 10 % FBS were considered to be derived from FBS because RPMI 1640 medium itself contains little protein according to the manufacturer's information. After reaction for 24 hr at 37°C, the mixture (0.5 ml) was centrifuged in a Microcon YM-3 (Millipore, Beverley, MA, USA). Concentrated DNP-conjugated BSA and DNP-conjugated FBS were washed three times, and the concentration of non-reacted DNCB in the flow-through fraction was measured by reversed-phase high-performance liquid chromatography (RP-HPLC) on a Shimadzu Class-VP system (Shimadzu Corporation, Kyoto, Japan) using a Capcell Pak C18 UG120 S-5 (4.6 mm I.D. x 150 mm) (Shiseido, Tokyo, Japan). DNP-conjugated BSA and DNP-conjugated FBS were also assayed by Western blotting using anti-dinitrophenol (anti-DNP) antibody (Cosmo Bio, Tokyo, Japan).

The concentration of DNCB conjugated to BSA was calculated by use of the following formula: conjugated DNCB (mg) = (DNCB content before incubation with BSA or RPMI 1640 medium with 10 % FBS) – (total content of unreacted DNCB in flow-through fraction).

Flow-cytometric analysis

Flow-cytometric analyses were performed with an EPICS XL-MCL System II (Beckman Coulter, Fullerton,

CA, USA). Dead cells were gated out by staining with propidium iodide (PI, 0.625 mg/ml). A total of 10,000 living cells was analyzed. When 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).

Measurement of CD86 expression by flow cytometry

THP-1 cells were seeded at 1 x 10^6 cells/ml. DNCB and DTNB were each dissolved in DMSO and added to the culture medium. The final concentration of the vehicle in the medium was under 0.2 % (v/v), which did not have any effect on cell growth (Ashikaga *et al.*, 2002). DNP-conjugated BSA or DNP-conjugated FBS was added directly to the culture medium. After 24 hr treatment with test chemicals, cells were washed with FACS buffer (phosphate-buffered saline (PBS) with 0.1 % BSA), then incubated with 50 ml of FITC-conjugated anti-human CD86 antibody (7 mg/ml) for 30 min on ice. After having been washed again with FACS buffer, cells were analyzed by flow cytometry. RFI was used as an indicator of CD86 expression and calculated as follows: RFI (%) = (MFI of sample-treated cells/ MFI of vehicle (DMSO) control cells or non-treated cells) x 100.

Analysis of cell-surface thiols and amines by flow cytometry

We used a nonpermeable thiol-reactive compound, Alexa Fluor 488 C_5 maleimide and a nonpermeable amine-reactive compound, Alexa Fluor 488 carboxylic acid succinimidyl ester, for detection of cell-surface thiols and amines. THP-1 cells were seeded at 1 x 10^6 cells/ml. DNCB and DTNB were each dissolved in DMSO and added to the culture medium. $NiSO_4$ and SDS were dissolved directly in the culture medium. After 2 hr treatment with test chemicals, THP-1 cells were recovered and washed with PBS twice, then incubated with 100 ml of Alexa Fluor 488 C_5 maleimide (10 mM) PBS solution or Alexa Fluor 488 carboxylic acid succinimidyl ester (10 mM) PBS solution for 30 min at 37°C. After having been washed again with PBS, the cells were analyzed by flow cytometry. RFI was calculated by use of the following formula: RFI (% of control) = (MFI of chemical-treated cells/ MFI of vehicle control cells or non-treated cells) x 100.

SDS-PAGE and Western blot analysis

Protein samples were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, USA). The membranes were incubated with Block Ace or Immuno Block (Dainippon Sumitomo Pharma, Osaka, Japan) for 60 min at room temperature (RT), and then with rabbit polyclonal antibodies to anti-phosphorylated p38 MAPK, anti-p38 MAPK and anti-DNP antibody for 60 min at RT. They were washed three times with Wash buffer (20 mM Tris-HCl (pH 7.6), 137 mM NaCl, 0.1 % Tween-20), then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 60 min at RT, and washed again three times with Wash buffer. Immunoreactive bands were detected by chemiluminescence measurement.

Analysis of phospho-p38 MAPK by Western blotting

After treatment with chemicals for 2 hr, phosphorylation of p38 MAPK was determined by Western blot analysis. Immunoblotting of phosphorylated p38 MAPK was performed using a p38 MAPK immunoblotting kit (Cell Signaling Technology, Beverly, MA, USA). Cells (1 x 10^6 cells/ml) were washed twice in cold PBS and resuspended in 50 ml of lysis buffer (1 % Nonidet P-40, 20 mM Tris-HCl (pH 8.0), 137 mM NaCl, 10 % glycerol, 2 mM ethylenediaminetetraacetic acid, 1 % protease inhibitor cocktail (Sigma-Aldrich), and 1 mM sodium orthovanadate). Nuclei and insoluble cell debris were removed by centrifugation at 14,000 x g for 10 min at 4°C. Postnuclear extracts were collected and used as total cell lysates. Total cell lysates were suspended in 2 x SDS sample buffer (313 mM Tris-HCl (pH 6.8), 10 % SDS, 2-ME, 50 % glycerol, and 0.01 % bromphenol blue) and heated at 95°C for 3 min. Protein samples were subjected to SDS-PAGE, and phosphorylated p38 MAPK and anti-p38 MAPK were detected by Western blotting. Rabbit polyclonal anti-phosphorylated p38 MAPK and anti-p38 MAPK antibodies were used as the primary antibodies.

Separation of cell-membrane proteins and cytoplasmic proteins from DNCB-treated THP-1, and Western blot analysis

Separation of cell-membrane proteins and cytoplasmic proteins from THP-1 cells treated with DNCB for 2 hr was performed according to the protocol of the Cytosol/Particulate Rapid Separation kit (BioVision, Mountain View, CA, USA). The separated cell-membrane proteins and cytoplasmic proteins were suspended in 2 x SDS sample buffer, and heated at 95°C for 3 min. Proteins (30 mg) were subjected to SDS-PAGE, and DNP-conjugated protein was detected by Western blotting. Rabbit polyclonal anti-DNP protein antibody was used as the primary antibody.

Measurement of MIP-1β production

Macrophage inflammatory protein-1β (MIP-1β) productions from THP-1 cells were measured as described previously (Hirota and Moro, 2006). Cultured supernatants of THP-1 cells treated with chemicals for 2 and 48 hr were recovered. The production of MIP-1β was assessed using human-specific cytokine ELISA kits (Quantikine, R&D systems, Minneapolis, MN, USA) according to the instructions of the manufacturer. The fold increase over the control was calculated by use of the following formula: MIP-1β production rate (% of control) = (MIP-1β production of chemical-treated cells/ MIP-1β production of vehicle control cells) x 100.

Analysis of endocytotic activity and cell-surface thiols by flow cytometry

We used a nonpermeable thiol-reactive compound, Alexa Fluor 568 C_5 maleimide, to label the cells. THP-1 cells were seeded at 1 x 10^6 cells/ml in culture medium. After 15 min treatment at 37°C with 150 mg/ml BSA Alexa Fluor 488 conjugate, THP-1 cells were recovered, washed with PBS three times, then incubated with 100 ml of Alexa Fluor 568 C_5 maleimide (10 mM) PBS solution for 30 min at 37°C. The cells were washed again with PBS, and analyzed by flow cytometry.

Statistical analysis

The statistical significance of differences in the RFI of cell surface thiols and amines, expressions of CD86 and MIP-1β production rate between non-treated THP-1 and chemical-treated THP-1 were analyzed using paired Student's *t* test.

RESULTS

CD86 expression on THP-1 cells treated with DNCB, DNP-conjugated BSA and DNP-conjugated FBS

In general, skin sensitizers have protein- and peptide-binding activity, or acquire their activity by metabolic activation. At first, we investigated whether hapten-treated THP-1 cells are activated by incorporation of hapten-conjugated protein or by hapten itself. We prepared DNP-conjugated BSA and DNP-conjugated FBS. Table 1 shows the amount of DNCB bound with BSA (A) or FBS (B), the amount of non-reacted DNCB, binding rate, amounts of BSA conjugated in the 50 mM and 15 mM DNP groups and amounts of 15 mM DNP-conjugated FBS in each experiment. THP-1 cells were treated with DNCB, DNP-conjugated BSA or DNP-conjugated FBS for 24 hr. When RFI of 150 % (control; 100 %) was

Table 1. CD86 expression on THP-1 treated with DNP-conjugated BSA

(A)

	DNCB conc.(μM)			Binding Rate (%)	DNP-conjugated BSA conc. (mg/ml)		BSA conc. (mg/ml)
	Before reaction (a)	non-reacted- (b)	conjugated- (a-b)		DNP (50 μM)-	DNP (15 μM)-	DNP (0 μM)-
Experiment 1.	494	195	299	61	1.9	0.83	1.9
Experiment 2.		334	160	32	3.9	1.3	3.9
Experiment 3.		322	172	35	3.3	1.1	3.3

(B)

	DNCB conc.(μM)			Binding Rate (%)	DNP-conjugated BSA conc. (mg/ml)	
	Before reaction (a)	non-reacted- (b)	conjugated- (a-b)		DNP (50 μM)-	DNP (15 μM)-
Experiment 4.	494	251	243	49	0.4	0.4
Experiment 5.		351	142	29	1.4	1.4

(A) Preparation of DNP-conjugated BSA. (B) Preparation of DNP-conjugated FBS. DNCB (494 mM (a)) DMSO solution was mixed with 10 mg/ml BSA or RPMI 1640 medium containing 10 % FBS. After reaction for 24 hr at 37 °C, non-reacted DNCB was separated from the reaction mixture and measured by HPLC (b). The DNP content in DNP-conjugated BSA or DNP-conjugated FBS was calculated by subtraction (a-b). Binding Rate (%) is the percent ratio of reacted DNCB (a-b) to total DNCB (a). The term 'conc.' represents concentrations of chemicals.

applied as the criterion as described in a previous report (Sakaguchi *et al.*, 2006), CD86 expression was induced only by direct treatment with 15 mM DNCB (Fig. 1).

Phosphorylation of p38 MAPK and changes of cell-surface thiols and amines on THP-1 cells treated with DNCB, NiSO4 and SDS

If THP-1 cells are activated by direct action of hapten as shown in Fig. 1, it is possible that the cell membrane, which is the first point of contact with haptens, triggers activation. Cell-membrane proteins, such as receptors, transport proteins and ion channels, are known to be sensors of environmental changes around cells. Further more, most proteins contain cysteine and lysine, which have nucleophilic side chains. We therefore compared changes in phosphorylation of intracellular p38 MAPK, an intracellular signaling molecule, with changes of cell membrane thiols and amines on THP-1 treated with sensitizers (DNCB and $NiSO_4$) and a non-sensitizer (SDS). DNCB and $NiSO_4$, but not SDS, induced a decrease of cell-surface thiols (Fig. 2A), and p38 MAPK were more highly phosphorylated after treatment with DNCB or $NiSO_4$ than after SDS (Fig. 2C). The change of cell-surface thiols appeared to be related to phosphorylation of p38 MAPK. On the other hand, cell-surface amines were decreased by DNCB or SDS, but not $NiSO_4$ (Fig. 2B).

Distribution of DNP-conjugated protein in DNCB-treated THP-1 cells

It was reported that exogenous non-permeable GSSG triggered a decrease of an intracellular glutathione (GSH), content and phosphorylation of p38 MAPK in U-937 cells (Filomeni *et al.*, 2003). GSSG is reported to be unable to permeate through the cell membrane (Filomeni *et al.*, 2003). On the other hand, it is not well established whether DNCB is membrane-permeable or not. Thus, we fractionated the cell-membrane proteins and cytoplasmic proteins in DNCB-treated THP-1, and detected DNCB-binding proteins by western blotting with anti-DNP protein antibody. As shown in Fig. 3, there were many DNCB-binding proteins among the cell membrane and cytoplasmic proteins. Though we expected that DNCB would affect only cell-membrane thiols, these data suggest that DNCB can activate THP-1 through modification of both cell-membrane and cytoplasmic proteins.

Activation of THP-1 cells treated with a membrane-impermeable thiol blocker, DTNB

As shown in Fig. 2, change of cell-surface thiols

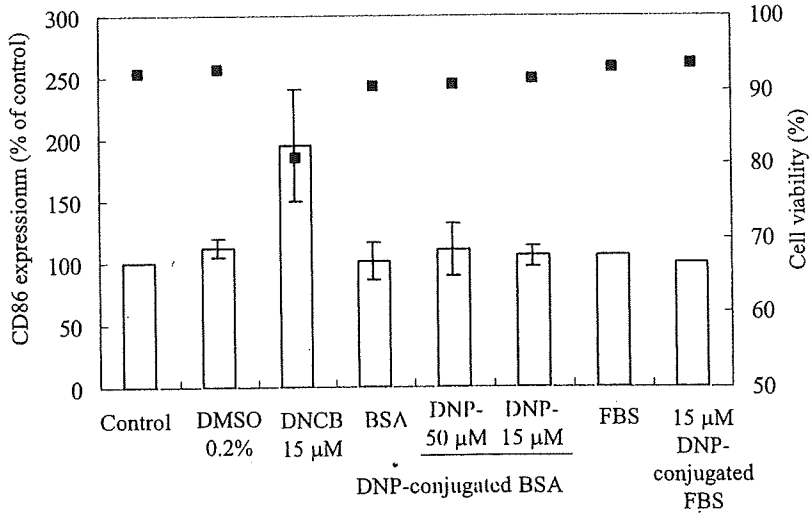

Fig. 1. THP-1 cells were treated with 15 mM DNCB, BSA, 50 mM DNP-conjugated BSA, 15 mM DNP-conjugated BSA, FBS and 15 mM DNP-conjugated FBS for 24 hr. The concentrations of BSA, 50 mM DNP-conjugated BSA, 15 mM DNP-conjugated BSA, FBS and 15 mM DNP-conjugated FBS are shown in Table 1(A) and (B). The concentration of BSA or FBS was the same with that of 50 mM DNP-conjugated BSA or 15 mM DNP-conjugated FBS, respectively. RFI values of CD86 expression were calculated as described in Materials and Methods. CD86 expression on THP-1 cells treated with DNCB (15 mM) for 24 hr was examined on the same day in each experiment. Each value of RFIs is the mean of ± S.D. of at three independent experiments. In the case of FBS and 15 mM DNP-conjugated FBS, results represented the mean of two independent experiments. Square dots indicated cell viability.

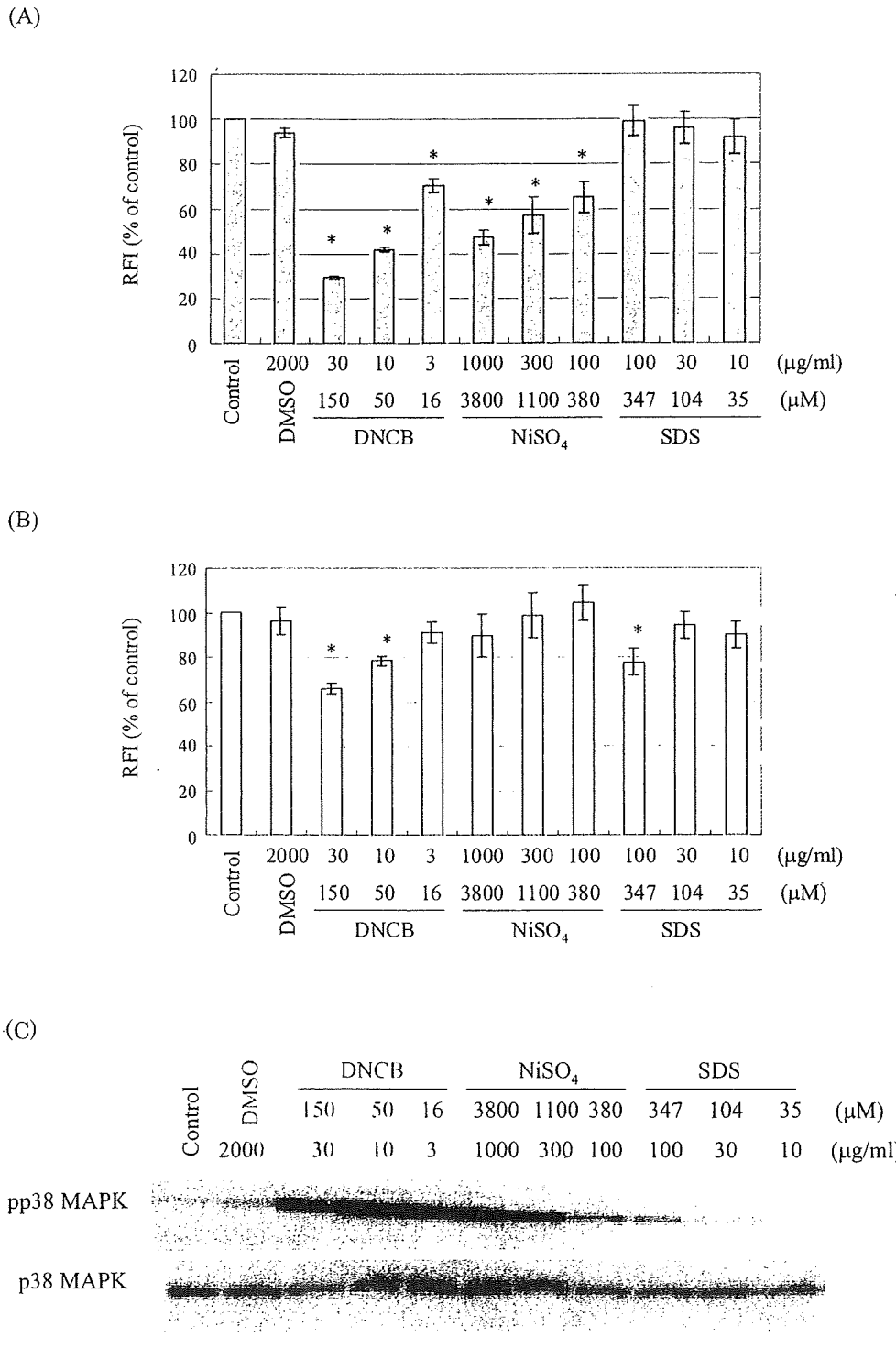

Fig. 2. Effect of chemicals on cell-surface thiols (A), amines (B) and phosphorylation of intracellular p38 MAPK (C) in THP-1 cells. THP-1 cells were exposed to test chemicals for 2 hr and cell-surface thiols and amines were measured by flow cytometry. Western blot analysis of phosphorylated p38 MAPK was performed using a p38 MAPK immunoblotting kit. Representative results of three independent sets are shown. Each value of RFIs is the mean of ± S.D. of at three independent experiments. *; $p < 0.05$.

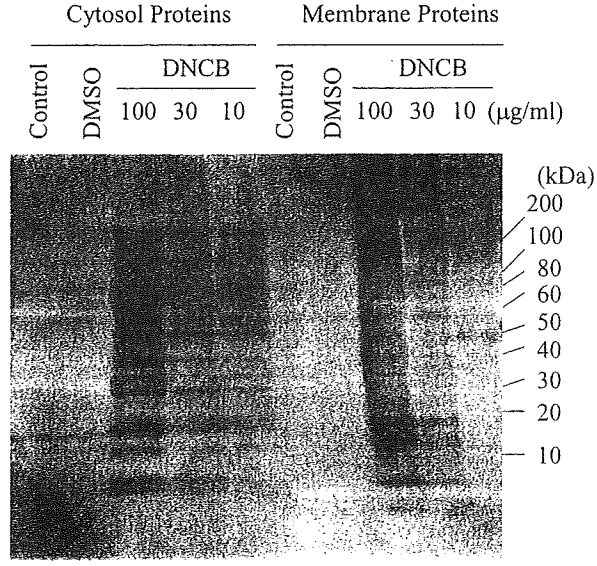

Fig. 3. Distribution of DNP-conjugated protein in DNCB-treated THP-1. THP-1 cells were exposed to DNCB for 2 hr. Separation of cell membrane proteins and cytoplasmic proteins of THP-1 cells was performed as described in Materials and Methods. The separated cell membrane proteins and cytoplasmic proteins (30 mg) were loaded on SDS-PAGE and DNP-conjugated protein was detected by Western blotting.

appeared to be related to phosphorylation of p38 MAPK, but haptens do not necessarily affect only cell-membrane proteins, if they are membrane-permeable, such as DNCB (Fig. 3). It is necessary to confirm cell activation by a membrane-impermeable thiol-modifying compound in order to prove the importance of cell-membrane thiols for cell activation. DTNB (5,5'-dithiobis (2-nitrobenzoic acid), Ellman's reagent) has been used for determining the thiols contents of cell-surface proteins because of its thiol-specific and membrane-impermeant properties (Reglinski *et al.*, 1988; Lawrence *et al.*, 1996; Laragione *et al.*, 2003). Therefore, we examined whether THP-1 cells are activated by modification of cell-surface thiols with DTNB by measuring cell-surface thiols, phosphorylation of p38 MAPK, CD86 expression and MIP-1β production, all of which have been reported to be candidate markers for *in vitro* sensitization methods (Ashikaga *et al.*, 2002; Hirota and Moro, 2006). DTNB treatment decreased cell-surface thiols and increased p38 MAPK phosphorylation (Fig. 4). CD86 expression was also augmented by 48 hr treatment with DTNB, and effect of DTNB suppressed by SB203580 (Fig. 5). Similarly, MIP-1β production was increased by treatment with DTNB for 2 and 48 hr (Fig. 6), and SB203580 suppressed increase of MIP-1β production in THP-1 cells treated with DTNB for 48 hr (Fig. 6B).

Endocytotic activity and cell-surface thiols of THP-1 cells

Receptor-mediated endocytosis has been reported as an early event of activation of LCs (Becker *et al.*, 1992; Girolomoni *et al.*, 1990). We thus investigated changes of cell-surface thiols by treatment with BSA Alexa Fluor 488 conjugate. As shown in Fig. 7, THP-1 displayed endocytic activity for BSA Alexa Fluor 488 conjugate. However, cell-surface thiols on THP-1 cells were unaffected.

DISCUSSION

According to the review by Ryan *et al.* (2005), changes in Langerhans cells as a result of exposure to chemical allergens include internalization of surface major histocompatibility complex (MHC) class II molecules via endocytosis (Becker *et al.*, 1992; Girolomoni *et al.*, 1990), induction of tyrosine phosphorylation (Kühn *et al.*, 1998), modulation of cell-surface markers (Aiba and Katz, 1990; Verrier *et al.*, 1999) and changes of cytokine expression (Enk and Katz, 1992).

We were interested in whether THP-1 cells are activated by hapten-conjugated exogenous protein, or by direct action of the hapten on the cells. In this *in vitro* study, we found that CD86 expression on THP-1 cells is augment-

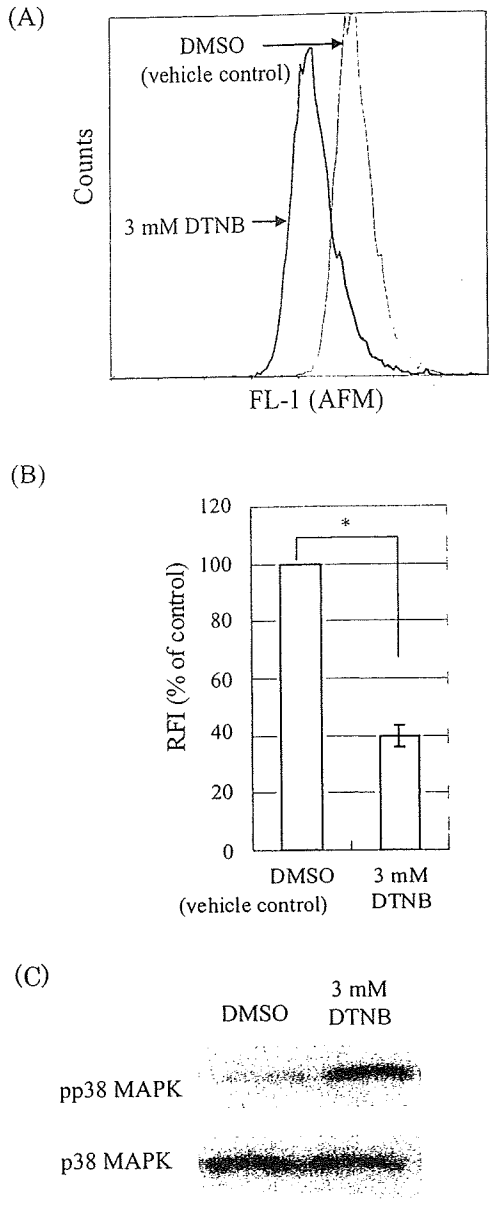

Fig. 4. Changes of cell-surface thiols and phosphorylation of intracellular p38 MAPK on THP-1 cells treated with a membrane-impermeable thiol blocker, DTNB. THP-1 cells were exposed to 3 mM DTNB for 2 hr and cell-surface thiols and phosphorylated p38 MAPK were analyzed as described in Materials and Methods. The flow-cytometric histograms (A) and RFI values (B) of THP-1 cells treated with 3 mM DTNB ((A) solid curve in (A)) and 2,000 mg/ml DMSO (vehicle control, shaded curves in (A)). Each value of RFIs in (B) is the mean of ± S.D. of three independent experiments. $*p < 0.05$. (C) Western blot analysis of intracellular phosphorylated p38 MAPK. Representative results of three independent sets are shown.

ed by treatment with DNCB, but not DNP-conjugated BSA or DNP-conjugated FBS. In *in vitro* studies, MHC class II internalization in hapten-treated Langerhans cells and CD86 expression in hapten-treated monocyte-derived dendritic cells were induced at sublethal concentrations of haptens (Becker *et al.*, 1992; Mizuashi *et al.*, 2005). However, we found that exogenous DNP-conjugated BSA and DNP-conjugated FBS, though not DNCB, were hardly toxic to THP-1 (Fig. 1). These observations supported the view that the trigger for activation of hapten-treated THP-1 cells is not hapten-conjugated protein.

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). Intracellular GSH, which is a tripeptide including cysteine, plays a key role in regulating the intracellular redox balance. According to the report by Mizuashi *et al.* (2005), the intracellular reduced/oxidized glutathione (GSH/GSSG) ratio was reduced by treatment with haptens. Although attention has mostly been focused on intracellular redox regulation, including transcription factors (Schreck *et al.*, 1991) and signaling molecules (Le *et al.*, 2000), cell-surface thiols also play an important role in lymphocytes (Sahaf *et al.*, 2003). Cell-surface proteins such as receptors and transporters can activate intracellular signal transduction, and cell membranes, including cell-surface proteins, are physically the first point of contact with haptens for cells. Cell-surface proteins that can be modified by oxidoreduction include ion channels and the N-methyl-D-aspartate (NMDA) receptor (Lipton *et al.*, 2002; Zeng *et al.*, 2003). Oxidation of cell-surface proteins induces activation of ERK via the epidermal growth factor receptor and activation of Src family protein tyrosine kinases by clustering these proteins through disulfide bond cross-linking (Nakashima *et al.*, 2002; Midwinter *et al.*, 2004). Furthermore, Usatyuk *et al.* (2006) demonstrated that 4-hydroxy-2-nonenal induced phosphorylation of p38 MAPK and protein modification via oxidative stress. We therefore hypothesized that small-molecular chemicals such as haptens directly activate cells, and that cell-surface proteins might act as sensors for cell activation. In this study, we demonstrated that cell-surface thiols, but not amines, were decreased by sensitizers (DNCB and $NiSO_4$), but not by a non-sensitizer, SDS, and also that p38 MAPK was more highly phosphorylated after DNCB and $NiSO_4$ treatment than after SDS treatment. The change of cell-surface thiols thus appeared to be associated with phosphorylation of p38 MAPK. DNCB and $NiSO_4$, which decreased at least cell-surface thiols, have been reported to aug-

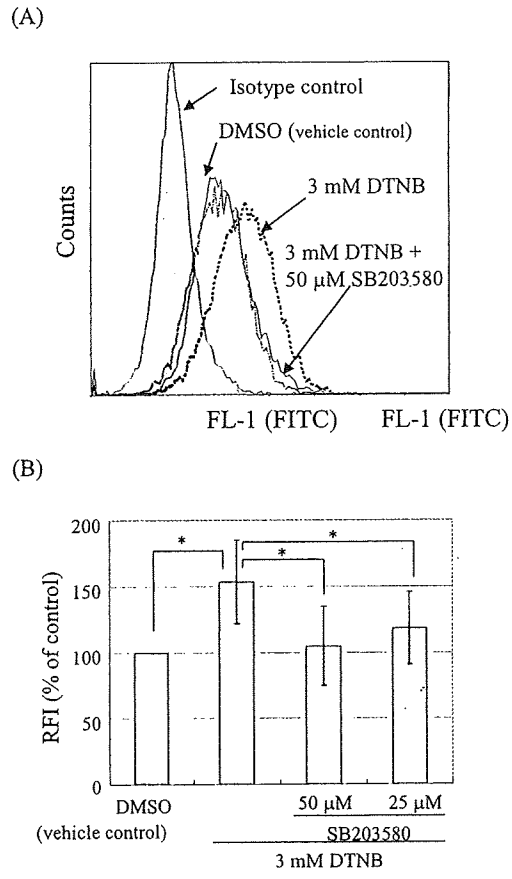

Fig. 5. CD86 expression on THP-1 cells treated with DTNB and effect of an inhibitor of the p38 MAPK pathway (SB203580). THP-1 cells were exposed to 3 mM DTNB with or without 50 mM SB203580 for 48 hr and CD86 expression was measured by flow cytometry (A) (2,000 mg/ml DMSO (vehicle control) (solid line), 3 mM DTNB (thick dotted line) and 3 mM DTNB + 50 mM SB203580 (thin dotted line)). The histogram of the isotype control is drawn with shaded curves. Representative results of three independent sets are shown. Each value of RFIs in (B) is the mean of ± S.D. of at five independent experiments. $*p < 0.05$.

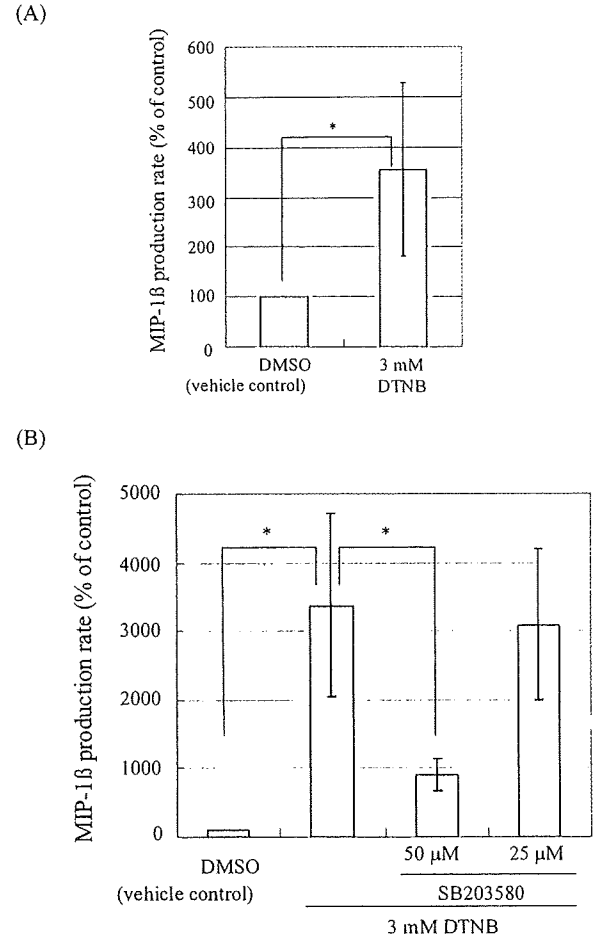

Fig. 6. MIP-1β production by THP-1 cells treated with DTNB and effect of an inhibitor of the p38 MAPK pathway (SB203580). THP-1 cells were exposed to 3 mM DTNB for 2 hr (A) and 48 hr (B) with or without 50 mM SB203580 for 48 hr and MIP-1β production was measured by ELISA. Each value of MIP-1β production is the mean of ± S.D. of at five independent experiments. $*p < 0.05$.

ment CD86 expression, whereas SDS, which decreased cell-surface amines, was reported not to augment CD86 expression (Sakaguchi *et al.*, 2006). These observations are consistent with the report of Becker *et al.* (2003), who showed that tyrosine phosphorylation induced by haptens was blocked in the presence of cysteine, but not lysine. Furthermore, Filomeni *et al.* (2003) reported that modulation of cell-surface thiols by exogenous, membrane-impermeable GSSG triggered a decrease of intracellular glutathione (GSH) content, activation of apoptosis signal-regulating kinase 1 (ASK1) and phosphorylation of p38 MAPK in U-937 cells. These observations and our data support the idea that changes of cell-surface thiols play a crucial role in the activation of the intracellular signal transduction pathway following hapten treatment. However, haptens may also have other effects. For example,

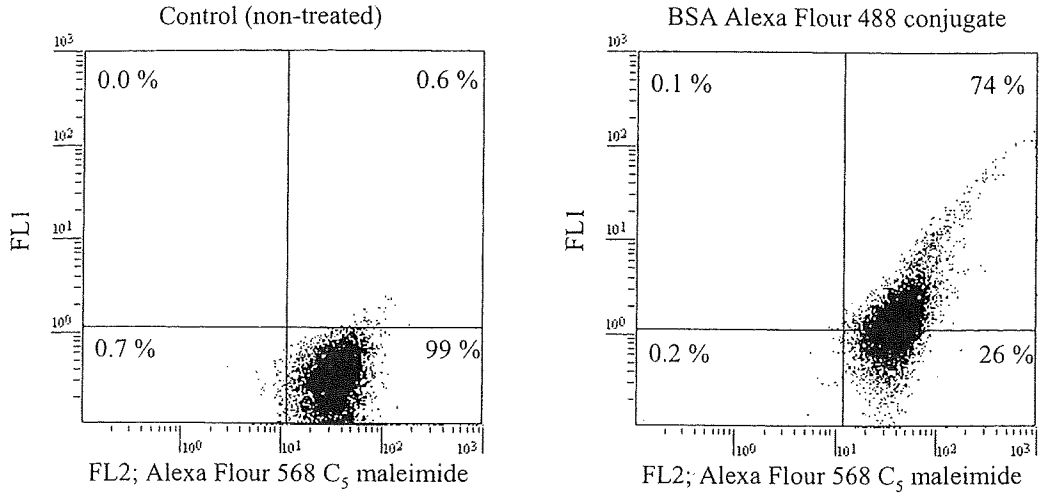

Fig. 7. Flow-cytometric dot-plots of THP-1 cells. THP-1 cells were exposed to 150 mg/ml BSA Alexa Fluor 488 conjugate for 15 min and cell-surface thiols were analyzed as described in Materials and Methods. Numbers in each quadrant represent cell percentages. Representative results of three independent sets are shown.

DNCB decreases not only cell-surface thiols, but also amines (Fig. 2) and DNP-conjugated protein of both cell-surface and cytoplasmic proteins were found in DNCB-treated THP-1 (Fig. 3). We found that THP-1 cells were activated through the p38 MAPK pathway, followed by augmentations of CD86 expression and MIP-1β production, upon treatment with a membrane-impermeable thiol blocker, DTNB. Considering that the increase of MIP-1β production by THP-1 cells was detected after 2 hr treatment with DTNB, activation of the signal transduction pathway appears to be an early response. These observations support the notion that cell-surface thiols are one of the triggers of activation through the p38 MAPK pathway in THP-1 cells treated with simple chemicals. This does not imply that hapten-conjugated proteins are not the sources of the peptides presented by antigen-presenting cells to T cells. We have shown here that THP-1 cells have endocytotic activity for BSA Alexa Fluor 488 conjugate independently of changes of cell-surface thiols. We previously reported the augmentation of cathepsin B gene expression in hapten-treated THP-1 cells, based on microarray analysis (Hirota and Moro, 2006). Cathepsin B is involved in degradation of protein Ag and generation of peptide-receptive MHC class II molecules (Fiebiger *et al.*, 2001). It seems likely that spontaneously endocytosed antigen, such as DNP-conjugated BSA and DNP-conjugated FBS, is degraded by intracellular proteases in activated THP-1 cells.

In this study, we focused on THP-1, a human monocytic cell line, but it would be interesting to know whether similar changes are found in antigen-presenting cells or not. Antigen-presenting cells have potent antigen-presenting functions and high endocytotic activity. Moreover, according to Filomeni *et al.* (2003), cytotoxic effects of GSSG on U-937, a monocytic cell line, differ from those on primary monocytes.

Recently, several groups have developed *in vitro* assays for predicting the sensitizing potentials of chemicals. Various biomarkers related to activation (maturation), migration and antigen-presentation of antigen-presenting cells have been used as targets. High sensitivity and an easy detection procedure are two of the most important requirements for *in vitro* assays. An assay detecting cell-surface thiols by flow cytometry would be straightforward. Thus, we are evaluating changes of cell-surface thiols as a candidate biomarker for an *in vitro* sensitization assay by using about thirty chemicals, including skin sensititers and non-sensitizers (Suzuki *et al.*, in press). We think cell-surface thiols might be a good candidate for detecting activation of cells treated with haptens.

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Oxidation of Cell Surface Thiol Groups by Contact Sensitizers Triggers the Maturation of Dendritic Cells

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p38 mitogen-activated protein kinase (MAPK) has a crucial role in the maturation of dendritic cells (DCs) by sensitizers. Recently, it has been reported that the oxidation of cell surface thiols by an exogenous impermeant thiol oxidizer can phosphorylate p38 MAPK. In this study, we examined whether sensitizers oxidize cell surface thiols of monocyte-derived DCs (MoDCs). When cell surface thiols were quantified by flow cytometry using Alexa fluor maleimide, all the sensitizers that we examined decreased cell surface thiols on MoDCs. To examine the effects of decreased cell surface thiols by sensitizers on DC maturation, we analyzed the effects of an impermeant thiol oxidizer, *o*-phenanthroline copper complex (CuPhen). The treatment of MoDCs with CuPhen decreased cell surface thiols, phosphorylated p38 MAPK, and induced MoDC maturation, that is, the augmentation of CD83, CD86, HLA-DR, and IL-8 mRNA, as well as the downregulation of aquaporin-3 mRNA. The augmentation of CD86 was significantly suppressed when MoDCs were pretreated with *N*-acetyl-L-cystein or treated with SB203580. Finally, we showed that epicutaneous application of 2,4-dinitrochlorobenzene on mouse skin significantly decreased cell surface thiols of Langerhans cells *in vivo*. These data suggest that the oxidation of cell surface thiols has some role in triggering DC maturation by sensitizers.

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INTRODUCTION

We have reported that murine Langerhans cells (LCs) upregulate their expression of class II major histocompatibility complex antigen and several co-stimulatory molecules, and consequently augment their antigen-presenting function after painting the skin with sensitizers, whereas chemicals that simply irritate the skin cannot induce this phenomenon (Aiba and Katz, 1990; Ozawa *et al.*, 1996). Consistent with these *in vivo* studies, using human monocyte-derived dendritic cells (MoDCs) or dendritic cells (DCs) derived from $CD34^+$ hematopoietic progenitor cells, we (Aiba *et al.*, 1997, 2000) and others (Coutant *et al.*, 1999; Tuschl *et al.*, 2000; Arrighi *et al.*, 2001; De Smedt *et al.*, 2001; Boisleve *et al.*, 2004; Staquet *et al.*, 2004) have shown *in vitro* that purified DCs respond to a variety of sensitizers, but not to irritants, such as benzalkonium chloride or sodium dodecyl sulfate (SDS), by significantly augmenting their expression of CD54, CD86, HLA-DR, and CCR7, and by increasing their production of proinflammatory cytokines. All these data show that sensitizers can induce the maturation of human DCs independent of the skin environment, suggesting that DCs play a crucial role in sensing simple chemicals that potentially sensitize naive T cells and induce allergic contact sensitivity.

In spite of these observations, however, it still remains undetermined how sensitizers with different chemical structures can activate DCs. Recently, using MoDCs, Arrighi *et al.* (2001), Brand *et al.* (2002), and we (Aiba *et al.*, 2003) have reported that several sensitizers,that is, 2,4-dinitrofluorobenzene (DNFB), 2,4-dinitrochlorobenzene (DNCB), and NiSO4, induce the phosphorylation of p38 mitogen-activated protein kinase (MAPK) and/or extracellular signal-regulated kinases (ERK), and that the phenotypic and functional changes induced by these sensitizers are suppressed by inhibitors of p38 MAPK or ERK. In addition, we have reported that $NiCl_2$ can activate NF-κB in MoDCs.

As for the mechanism by which p38 MAPK is activated by sensitizers in spite of the lack of proven specific receptors, we have reported the role of the redox imbalance in the maturation of DCs induced by sensitizers (Mizuashi *et al.*, 2005); that is, we have shown that all the sensitizers decreased the intracellular reduced/oxidized glutathione ratio (GSH/GSSG ratio) in MoDCs, and that this was accompanied by the phosphorylation of p38 MAPK. Moreover, treatment with *N*-acetyl-L-cystein (NAC), which suppressed the reduction of the GSH/GSSG ratio in MoDCs,

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Abbreviations: Ab, antibody; AQP3, aquaporin-3; CuPhen, o-phenanthroline copper complex; DC, dendritic cell; DNCB, 2,4-dinitrochlorobenzene ERK, extracellular signal-regulated kinases; GSH, reduced glutathione GSSG, oxidized glutathione; JNK, c-Jun N-terminal kinase; LC, Langerhans cell; MAPK, mitogen-activated protein kinase; MoDC, monocyte-derived dendritic cell; NAC, N-acetyl-L-cystein; SAPK, stress-activated protein kinase

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abrogated both the phosphorylation of p38 MAPK and the augmentation of CD86 expression by MoDCs.

Recently, it has been reported that oxidation of cell surface thiols in human monocyte cell line, U937, decreases the intracellular GSH content and phosphorylates p38 MAPK (Filomeni *et al.*, 2003). In addition, we have also shown that the oxidation of cell surface thiols can stimulate human monocyte cell line, THP-1, to show the phenotypic changes that characterize the maturation of DCs, for example, the augmentation of CD86 expression and MIP-1β production, and that these phenotypic changes are mainly mediated by the activation of p38 MAPK (Hirota *et al.*, 2009). Moreover, by examining the effects on cell surface thiols of THP-1 by 35 skin sensitizers and 17 non-sensitizers, we have found that most sensitizers significantly reduce cell surface thiols, whereas non-sensitizers except for Tween80 and resorcinol do not (Suzuki *et al.*, 2009). Therefore, we hypothesized that sensitizers oxidize the cell surface thiols of MoDCs, leading to the phosphorylation of p38 MAPK and their maturation. Therefore, in this study, we showed whether sensitizers decrease the cell surface thiols. Next, we showed that an impermeant thiol oxidizer, *o*-phenanthroline copper complex (CuPhen), decreases the cell surface thiols of MoDCs and phosphorylates p38 MAPK, and induces MoDC maturation. Finally, we examined whether epidermal LCs also decrease the cell surface thiols after hapten application on the skin.

RESULTS

All contact sensitizers, but not non-sensitizers, at sublethal concentrations, decreased cell surface thiols of MoDCs and increased their phosphorylated p38 MAPK

As we have reported that some of the sensitizers decreased the intracellular GSH/GSSG ratio in MoDCs, phosphorylated p38 MAPK, and augmented their CD86 expression, in this study, we examined whether sensitizers decrease the cell surface thiols on MoDCs by flow cytometry using an Alexa fluor C_5 maleimide (Invitrogen, Carlsbad, CA), a specific impermeant thiol-reactive compound able to covalently bind to membrane surface cysteines (Figure 1). When we treated MoDCs with various sensitizers, a non-sensitizer, SDS, and a representative danger signal, ATP, for 2 hours, all the sensitizers decreased the exofacial thiol groups in a dose-dependent manner, whereas SDS or ATP did not significantly alter them. In addition, only the sensitizers phosphorylated p38 MAPK of MoDCs. Again, SDS or ATP did not significantly increase phosphorylated p38 MAPK. When we examined the cell viability 2 hours after chemical treatment, it was more than 80% in the concentration used in this experiment. To further clarify the relationship between cell surface thiol expression and phosphorylation of p38 MAPK, we examined the correlation between the relative fluorescence intensity of cell surface thiols stained by Alexa fluor C_5 maleimide and the relative fluorescence intensity of phospho-p38 MAPK stained by anti-phospho-p38 MAPK antibody (Ab) in MoDCs treated with various concentrations of chemicals. The results showed a significant inverse correlation between them.

An impermeant thiol oxidizer, CuPhen, decreased cell surface thiols of MoDCs, phosphorylated p38 MAPK, and activated MoDCs

Ciriolo *et al.* (1993) and Filomeni *et al.* (2003) reported that reducing/oxidizing impermeant agents could affect the intracellular redox state by translocation of their equivalents from the external to the intracellular space. As our current studies revealed that sensitizers decreased the cell surface thiols, we next examined whether the oxidation of cell surface thiols affected the phosphorylation of p38 MAPK of MoDCs and activated them. In this study, we used CuPhen as an impermeant thiol oxidizer (Duncan and Lawrence, 1989; Lawrence *et al.*, 1996). When MoDCs were treated with three different concentrations of CuPhen, that is, 1, 2, and 3 μM, for different time periods, those treated with 2 and 3 μM of CuPhen decreased their cell surface thiol group from 15 minutes after treatment, reaching to a statistically significant level at 2 hours after treatment (Figure 2a and b). Moreover, 2 and 3 μM of CuPhen phosphorylated p38 MAPK 1 hour after treatment, whereas 3 μM of CuPhen phosphorylated it 2 hours after treatment (Figure 2c). It was also confirmed by flow cytometry; that is, when we examined phosphorylated p38 MAPK of MoDCs 2 hours after CuPhen treatment by flow cytometry, 3 μM of CuPhen induced a right shift of the single peak of phosphorylated p38 MAPK in four independent experiments. In addition, both the concentrations of CuPhen also phosphorylated p42/44 ERKs (Figure 2d), although neither concentrations of CuPhen significantly affected phosphorylation of stress-activated protein kinase/c-Jun *N*-terminal kinase (SAPK/JNK) (Figure 2e) or IκB (Figure 2f). When we examined the cell viability 2 hours after chemical treatment, it was more than 80% in the concentration used in this experiment. After 24 hours culture, 2 and 3 μM of CuPhen significantly augmented HLA-DR, CD86, and CD83 expression, although 3 μM of CuPhen significantly decreased the cell viability (Figure 3a). As for maturation markers of MoDCs, other than these phenotypic changes, we also examined IL-8 and aquaporin-3 (AQP3) mRNA expression by MoDCs treated with CuPhen, as de Baey and Lanzavecchia (2000) reported that AQP3 mRNA expression is downregulated during DC maturation. Consistent with the augmentation of CD86 expression, 2 and 3 μM of CuPhen significantly augmented IL-8 mRNA and suppressed AQP3 mRNA expression by MoDCs (Figure 3b).

NAC blocked the reduction of the cell surface thiols of MoDCs induced by CuPhen and suppressed DC maturation

Next, we pretreated MoDCs with NAC 30 minutes before the treatment with CuPhen. NAC is a well-known thiol antioxidant, and suppresses JNK, p38 MAPK, redox-sensitive activating protein-1, and NF-κB transcription factor activities (reviewed by Zafarullah *et al.* (2003)). The treatment of MoDCs with NAC significantly increased the cell surface thiols and suppressed their reduction induced by the treatment with CuPhen (Figure 4a). Consistent with the effects on the cell surface thiols, NAC suppressed the phosphorylation of p38 MAPK (Figure 4b and c) and MoDC maturation

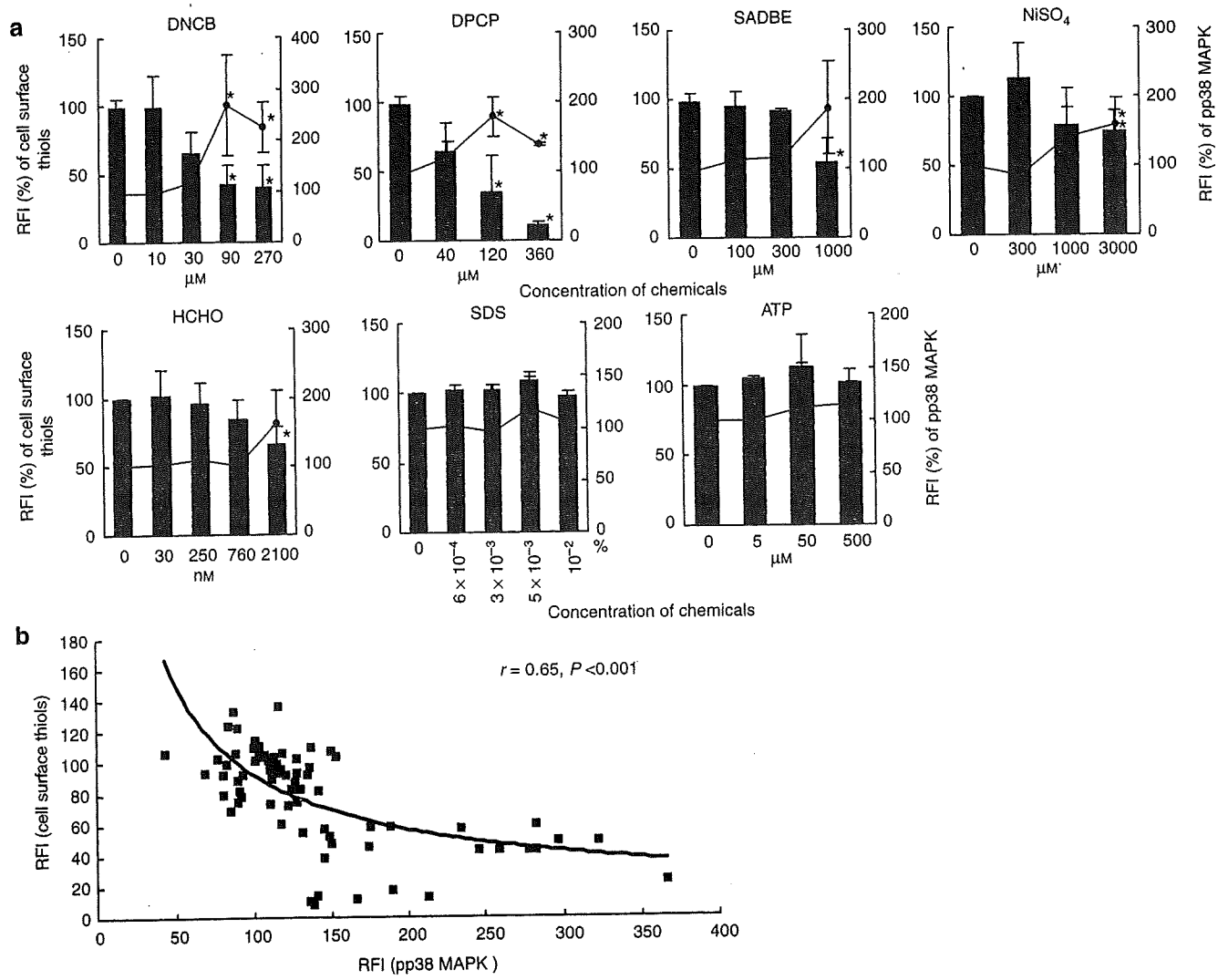

Figure 1. Sensitizers decrease cell surface thiols and phosphorylate p38 MAPK. MoDCs were treated with various concentrations of contact sensitizers, SDS, or ATP for 2 hours. The cell surface thiols were quantified by flow cytometry using thiol-reactive Alex Fluor 488 C5 maleimide. In addition, the intracellular expression of phospho-p38 MAPK (pp38 MAPK) was analyzed by flow cytometry using PE-conjugated anti-pp38 MAPK. (**a**) The mean ± SEM of RFI for cell surface thiols and pp38 MAPK from three to five independent experiments is shown. Asterisks indicate significance (*$P<0.05$) for the difference between stimulated cells and the vehicle-treated control. (**b**) RFIs for cell surface thiols and pp38 MAPK from three to five independent experiments are plotted and analyzed by Pearson's correlation coefficient. RFI, relative fluorescence intensity.

induced by CuPhen (Figure 4d). Interestingly, NAC also rescued the reduction of the cell viability by the treatment with 2 μM CuPhen.

SB203580 suppressed the DC maturation induced by CuPhen

Next, we treated MoDCs with SB203580 to confirm that the stimulation with CuPhen is really mediated through p38 MAPK. The treatment of MoDCs with SB203580 significantly suppressed the augmentation of CD86 expression by CuPhen (Figure 5).

Murine epidermal LCs also decreased the cell surface thiols after DNCB painting

These *in vitro* studies suggest that the reduction of the cell surface thiols of DCs treated with sensitizers induced the signal for DC maturation. We next examined whether epicutaneous application of a representative hapten, DNCB, induced the reduction of the cell surface thiols of epidermal LC. At 2 hours after DNCB painting, epidermal LC that were identified as epidermal $CD86^+$ cells decreased the cell surface thiols (Figure 6).

DISCUSSION

Recently, Tanaka *et al.* (2005) have reported that cell surface thiols other than glutathione regulate signal transduction, leading to phosphorylation of protein kinase B and activation of endothelial nitric oxide synthase. On the other hand, Filomeni *et al.* (2003) have suggested that the oxidation of cell surface thiols by exogenous impermeant GSSG can phosphorylate p38 MAPK. As it has been shown by several researchers that sensitizers phosphorylate p38 MAPK and that the DC maturation induced by sensitizers is at least partly mediated by activated p38 MAPK (Arrighi *et al.*, 2001; Brand *et al.*, 2002; Aiba *et al.*, 2003), in this study, we examined