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

Morihiko Hirota¹, Mie Suzuki¹, Shigenobu Hagino¹, Saori Kagatani², Yoshinori Sasaki²,
Setsuya Aiba² and Hiroshi Itagaki¹

¹Quality Assessment Center, Shiseido Co., Ltd., 2-12-1 Fukuura, Kanazawa-ku, Yokohama-shi, Kanagawa
236-8643, Japan

²Department of Dermatology, Tohoku University Graduate School of Medicine, 1-1 Seiryō-machi, Aoba-ku, Sendai,
Miyagi 980-8574, Japan

(Received September 26, 2008; Accepted December 8, 2008)

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 (NiSO₄) 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-1 β (MIP-1 β) 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-1 β , 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 (NiCl₂) and 2,4-dinitrochlorobenzene (DNCB), but not to irritants, such as sodium dodecyl sul-

fate (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 pPD. The THP-1 cell line was reported to be a good candidate indicator for *in*

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

p38 mitogen-activated protein kinase (MAPK) plays a crucial role 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 C₅ maleimide, Alexa Fluor 568 C₅ maleimide, nonpermeable amine reactive compound Alexa Fluor 488 carboxylic acid suc-

cinimidyl 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 % CO₂ incubator. Cells were passaged by addition of fresh medium twice a week and cell density was maintained between 0.1 and 0.5 × 10⁶ 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, Beverly, 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. × 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 1×10^6 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: $\text{RFI (\%)} = (\text{MFI of sample-treated cells} / \text{MFI of vehicle (DMSO) control cells or non-treated cells}) \times 100$.

Analysis of cell-surface thiols and amines by flow cytometry

We used a nonpermeable thiol-reactive compound, Alexa Fluor 488 C₅ 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 1×10^6 cells/ml. DNCB and DTNB were each dissolved in DMSO and added to the culture medium. NiSO₄ 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 C₅ 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: $\text{RFI (\% of control)} = (\text{MFI of chemical-treated cells} / \text{MFI of vehicle control cells or non-treated cells}) \times 100$.

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 (1×10^6 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 $14,000 \times g$ 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 % bromophenol 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-1 β production

Macrophage inflammatory protein-1 β (MIP-1 β) 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-1 β 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-1 β production rate (% of control) = (MIP-1 β production of chemical-treated cells/ MIP-1 β 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 C₅ maleimide, to label the cells. THP-1 cells were seeded at 1×10^6 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 C₅ 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-1 β 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.(μ M)			Binding Rate (%)	DNP-conjugated BSA conc. (mg/ml)		BSA conc. (mg/ml)
	Before reaction (a)	non-reacted- (b)	conjugated- (a-b)		DNP (50 μ M)-	DNP (15 μ M)-	DNP (0 μ M)-
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.(μ M)			Binding Rate (%)	DNP-conjugated BSA conc. (mg/ml)	
	Before reaction (a)	non-reacted- (b)	conjugated- (a-b)		DNP (50 μ M)-	DNP (15 μ M)-
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 NiSO₄) and a non-sensitizer (SDS). DNCB and NiSO₄, but not SDS, induced a decrease of cell-surface thiols (Fig. 2A), and p38 MAPK were more highly phosphorylated after treatment with DNCB or NiSO₄ 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 NiSO₄ (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 $\pm$ 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.

(A)

(B)

(C)

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 $\pm$ S.D. of at three independent experiments. *; $p < 0.05$.

Modification of cell-surface thiols elicits activation of THP-1 cells

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-1 β 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-1 β production was increased by treatment with DTNB for 2 and 48 hr (Fig. 6), and SB203580 suppressed increase of MIP-1 β 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 endocytotic 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 RFI in (B) is the mean of $\pm$ 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-

Modification of cell-surface thiols elicits activation of THP-1 cells

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 $\pm$ 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-

Fig. 6. MIP-1 β 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-1 β production was measured by ELISA. Each value of MIP-1 β production is the mean of $\pm$ S.D. of at five independent experiments. * p < 0.05.

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-1 β production, upon treatment with a membrane-impermeable thiol blocker, DTNB. Considering that the increase of MIP-1 β 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

er 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 sensitizers 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.

ACKNOWLEDGMENT

We thank Richard Steele for critical review of the manuscript.

REFERENCES

- Aiba, S. and Katz, S.I. (1990): Phenotypic and functional characteristics of *in vivo*-activated Langerhans cells. *J. Immunol.*, **145**,

Modification of cell-surface thiols elicits activation of THP-1 cells

- 2791-2796.
- Aiba, S., Terunuma, A., Manome, H. and Tagami, H. (1997): Dendritic cells differently respond to haptens and irritants by their production of cytokines and expression of co-stimulatory molecules. *Eur. J. Immunol.*, **27**, 3031-3038.
- Aiba, S. and Tagami, H. (1998): Dendritic cell activation induced by various stimuli, e.g. exposure to microorganisms, their products, cytokines, and simple chemicals as well as adhesion to extracellular matrix. *J. Dermatol. Sci.*, **20**, 1-13.
- Aiba, S., Manome, H., Nakagawa, S., Mollah, Z.U., Mizuashi, M., Ohtani, T., Yoshino, Y. and Tagami, H. (2003): p38 Mitogen-activated protein kinase and extracellular signal-regulated kinases play distinct role in the activation of dendritic cells by two representative haptens, NiCl₂ and 2,4-dinitrochlorobenzene. *J. Invest. Dermatol.*, **120**, 390-399.
- Ashikaga, T., Hoya, M., Itagaki, H., Katsumura, Y. and Aiba, S. (2002): Evaluation of CD86 expression and MHC class II molecule internalization in THP-1 human monocyte cells as predictive endpoints for contact sensitizers. *Toxicol. In Vitro*, **16**, 711-716.
- Ashikaga, T., Yoshida, Y., Hirota, M., Yoneyama, K., Itagaki, H., Sakaguchi, H., Miyazawa, M., Ito, Y., Suzuki, H. and Toyoda, H. (2006): Development of an *in vitro* skin sensitization test using human cell lines: The human Cell Line Activation Test (h-CLAT) I. Optimization of the h-CLAT protocol. *Toxicol. In Vitro*, **20**, 767-773.
- Azam, P., Peiffer, J.L., Chamousset, D., Tissier, M.H., Bonnet, P. A., Vian, L., Fabre, I. and Ourlin, J.C. (2006): The cytokine-dependent MUTZ-3 cell line as an *in vitro* model for the screening of contact sensitizers. *Toxicol. Appl. Pharmacol.*, **212**, 14-23.
- Becker, D., Kolde, G., Reske, K. and Knop, J. (1994): An *in vitro* test for endocytotic activation of murine epidermal langerhans cells under the influence of contact allergens. *J. Immunol. Method*, **169**, 195-204.
- Becker, D., Mohammadzadeh, M., Reske, K. and Knop, J. (1992): Increased level of intracellular MHC class II molecules in murine Langerhans cells following *in vivo* and *in vitro* administration of contact allergens. *J. Invest. Dermatol.*, **99**, 545-549.
- Becker, D., Valk, E., Zahn, S., Brand, P. and Knop, J. (2003): Coupling of contact sensitizers to thiol groups is a key event for the activation of monocytes and monocyte-derived dendritic cells. *J. Invest. Dermatol.*, **120**, 233-238.
- Coutant, K.D., de Fraissinette, A.B., Cordier, A. and Ulrich, P. (1999): Modulation of the activity of human monocyte-derived dendritic cells by chemical haptens, a metal allergen, and a staphylococcal superantigen. *Toxicol. Sci.*, **52**, 189-198.
- Enk, A.H. and Katz, S.I. (1992): Early molecular events in the induction phase of contact sensitivity. *PNAS*, **89**, 398-1402.
- Filomeni, G., Rotilio, G. and Ciriolo, M.R. (2003): Glutathione disulfide induces apoptosis in U-937 cells by a redox-mediated p38 MAP kinase pathway. *FASEB J.*, **17**, 64-66.
- Fiebiger, E., Meraner, P., Weber, E., Fang, I.F., Stingl, G., Ploegh, H. and Maurer, D. (2001): Cytokines regulate proteolysis in major histocompatibility complex class II-dependent antigen presentation by dendritic cells. *J. Exp. Med.*, **193**, 881-892.
- Gerberick, G.F., Vassallo, J.D., Bailey, R.E., Chaney, J.G., Morrall, S.W. and Lepoittevin, J.P. (2004): Development of a peptide reactivity assay for screening contact allergens. *Toxicol. Sci.*, **81**, 332-343.
- Girolmoni, G., Cruz, P.D.Jr. and Bergstresser, P.R. (1990): Internalization and acidification of surface HLA-DR molecules by epidermal Langerhans cells: a paradigm for antigen processing. *J. Invest. Dermatol.*, **94**, 753-760.
- Hirota, M. and Moro, O. (2006): MIP-1beta, a novel biomarker for *in vitro* sensitization test using human monocytic cell line. *Toxicol. In Vitro*, **20**, 736-742.
- Huletter, B.C., Rowden, G., Ryan, C.A., Lawson, C.M., Dawes, S.M., Ridder, G.M. and Gerberick G.F. (2001): Cytokine induction of a human acute myelogenous leukemia cell line (KG-1) to a CD1a⁺ dendritic cell phenotype. *Arch. Dermatol. Res.*, **293**, 147-158.
- Kühn, U., Brand, P., Willemsen, J., Jonuleit, H., Enk, A.H., van Brandwijk-Petershans, R., Saloga, J., Knop, J. and Becker, D. (1998): Induction of tyrosine phosphorylation in human MHC class II-positive antigen-presenting cells by stimulation with contact sensitizers. *J. Immunol.*, **160**, 667-673.
- Laragione, T., Bonetto, V., Casoni, F., Massignan, T., Bianchi, G. and Gianazza, E. and Ghezzi, P. (2003): Redox regulation of surface protein thiols: identification of integrin alpha-4 as a molecular target by using redox proteomics. *PNAS*, **100**, 14737-14741.
- Lawrence, D.A., Song, R. and Weber, P. (1996): Surface thiols of human lymphocytes and their changes after *in vitro* and *in vivo* activation. *J. Leukocyte. Biol.*, **60**, 611-618.
- Le, F., Stomski, F., Woodcock, J.M., Lopez, A.F. and Gonda, T.J. (2000): The role of disulfide-linked dimerization in interleukin-3 receptor signaling and biological activity. *J. Biol. Chem.*, **275**, 5124-5130.
- Lipton, S.A., Choi, Y.B., Takahashi, H., Zhang, D., Li, W., Godzik, A. and Bankston, L.A. (2002): Cysteine regulation of protein function--as exemplified by NMDA-receptor modulation. *Trends Neurosci.*, **25**, 474-480.
- Midwinter, R.G., Peskin, A.V., Vissers, M.C. and Winterbourn, C.C. (2004): Extracellular oxidation by taurine chloramine activates ERK via the epidermal growth factor receptor. *J. Biol. Chem.*, **279**, 32205-32211.
- Mizuashi, M., Ohtani, T., Nakagawa, S. and Aiba, S. (2005): Redox imbalance induced by contact sensitizers triggers the maturation of dendritic cells. *J. Invest. Dermatol.*, **124**, 579-586.
- Nakashima, I., Kato, M., Akhand, A.A., Suzuki, H., Takeda, K., Hossain, K. and Kawamoto, Y. (2002): Redox-linked signal transduction pathways for protein tyrosine kinase activation. *Antioxid. Redox Signal.*, **4**, 517-531.
- Reglinski, J., Hoey, S., Smith, W.E. and Sturrock, R.D. (1988): Cellular response to oxidative stress at sulfhydryl group receptor sites on the erythrocyte membrane. *J. Biol. Chem.*, **263**, 12360-12366.
- Ryan, C.A., Gerberick, G.F., Gildea, L.A., Huletter, B.C., Betts, C.J., Cumberbatch, M., Dearman, R.J. and Kimber, I. (2005): Interactions of contact allergens with dendritic cells: Opportunities and challenges for the development of novel approaches to hazard assessment. *Toxicol. Sci.*, **88**, 4-11.
- Sahaf, B., Heydari, K., Herzenberg, L.A. and Herzenberg, L.A. (2003): Lymphocyte surface thiol levels. *PNAS*, **100**, 4001-4005.
- Sakaguchi, H., Ashikaga, T., Miyazawa, M., Yoshida, Y., Ito, Y., Yoneyama, K., Hirota, M., Itagaki, H., Toyoda, H. and Suzuki, H. (2006): Development of an *in vitro* skin sensitization test using human cell lines; human Cell Line Activation Test (h-CLAT). II. An inter-laboratory study of the h-CLAT. *Toxicol. In Vitro*, **20**, 774-784.
- Schreck, R., Rieber, P. and Baeuerle, P.A. (1991): Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-kappa B transcription factor and HIV-1. *EMBO J.*, **10**, 2247-2258.
- Suzuki, M., Hirota, M., Hagino, S., Itagaki, H. and Aiba, S. (2009):

- Evaluation of changes of cell-surface thiols as a new biomarker for *in vitro* sensitization test. *Toxicol. In Vitro.* (in press).
- Usatyuk, P.V., Parinandi, N.L. and Natarajan, V., (2006): Redox regulation of 4-hydroxy-2-nonenal-mediated endothelial barrier dysfunction by focal adhesion, adherens, and tight junction proteins. *J. Biol. Chem.*, **281**, 35554-35566.
- Verrier, A.C., Schmitt, D. and Staquet, M.J. (1999): Fragrance and contact allergens *in vitro* modulate the HLA-DR and E-cadherin expression on human epidermal Langerhans cells. *Int. Arch. Allergy Immunol.*, **120**, 56-62.
- Yoshida, Y., Sakaguchi, H., Ito, Y., Okuda, M. and Suzuki, H. (2003): Evaluation of the skin sensitization potential of chemicals using expression of co-stimulatory molecules, CD54 and CD86, on the naïve THP-1 cell line. *Toxicol. In Vitro*, **17**, 221-228.
- Zeng, X.H., Xia, X.M. and Lingle, C.J. (2003): Redox-sensitive extracellular gates formed by auxiliary beta subunits of calcium-activated potassium channels. *Nat. Struct. Biol.*, **10**, 448-454.