INTRODUCTION

Skin sensitization is a cell-mediated, delayed-type hypersensitivity immune response induced by low-molecular-weight compounds called haptens. Dendritic cells, including Langerhans cells, are potent antigen-presenting cells and play an important role in the induction of skin sensitization by simple chemicals (Aiba and Tagami, 1998). Activation by haptens has been observed in cultured human monocyte-derived dendritic cells (MoDCs); these cells respond in vitro to haptens such as nickel chloride (NiCl2) and 2,4-dinitrochlorobenzene (DNCB) by significantly augmenting their expression of CD86, CD54, HLA-DR and CCR7 (Aiba et al., 1997; Coutant et al., 1999; Boislève et al., 2004). Furthermore, mitogen-activated protein kinases (MAPKs), such as p38 mitogen-activated protein kinase (p38 MAPK), extracellular signal-regulated kinases (ERK) and c-jun N-terminal kinase (JNK), play crucial rules in the augmentation of CD86 and CCR7 expression on dendritic cells treated with haptens (Aiba et al., 2003; Boislève et al., 2004).

On the other hand, the reduction-oxidation (redox) state of cells reflects the balance between the levels of oxidizing and reducing equivalents. The redox balance is important for cell activation. It was demonstrat-
ed that intracellular redox imbalance is a signaling mediator up-stream of p38 MAPK in hapten-treated MoDCs and THP-1 cells (Mizuashi et al., 2005). Filomeni et al. (2003) reported that oxidation of exofacial membrane thiol groups by exogenous non-permeable GSSG triggered a decrease of intracellular glutathione (GSH) content, phosphorylation of p38 MAPK and apoptosis in U-937 cell lines. Becker et al. (2003) reported that coupling of sensitizers to thiol groups is a key event for the activation of monocytes and MoDCs. Furthermore, we demonstrated that a decrease of cell-surface thiols is a trigger of activation of intracellular signal transduction in hapten-treated MoDCs and THP-1 cells (Hirota et al., 2009; Kagatani et al., 2010), and that the level of cell-surface thiols on THP-1 cells decreases in response to treatment with many kinds of hapten (Suzuki et al., 2009). On the other hand, increase of cell-surface thiols on cells has been observed in response to N-acetyl-L-cysteine (NAC), a thiol antioxidant, and mitogens such as concanavalin A (Con A) and phytohemagglutinin (PHA-L) (Laragione et al., 2003; Lawrence et al., 1996). However, the effect of cell-surface thiol-increasing chemicals on intracellular signal transduction in cells is unknown.

From such a viewpoint, we focused on the increase in cell-surface thiols in THP-1 cell lines treated with diphenyldicyclopropenone (DPCP). DPCP is used as a drug to treat alopecia areata, and is a potent sensitizer (Holzer et al., 2005). 2-Mercaptoethanol (2-ME) (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 the addition of fresh medium twice a week and cell density was maintained between 0.1 and 0.5 x 10^6 cells/ml.

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized fresh leukocyte-enriched buffy coats from different donors using Lymphoprep (Nycomed Pharma As, Oslo, Norway). After several washes with phosphate-buffered saline (PBS), 1 x 10^6 PBMC were treated with 150 µl of CD14 microbeads in 600 µl of PBS supplemented with 1% bovine serum albumin (BSA) and 5 mM EDTA (MACS buffer) at 4°C for 30 min. The cells coated with CD14 microbeads were washed with MACS buffer, then separated by a magnetic cell separator, MACS (Miltenyi Biotech), according to the manufacturer’s protocol. Before culturing, we examined the percentage of CD14^+ cells in these preparations by flow cytometry (FAC-Scalibur using CellQuest software (Becton Dickinson, San Jose, CA, USA)). Only cell specimens containing more than 90% CD14^+ cells were used in the experiments. This study was approved by the ethics committee of Shiseido Research Center, Yokohama, Japan and the ethics committee of Tohoku University Graduate School of Medicine, Sendai, Japan, and adhered to the guidelines set forth by the Helsinki protocol. All the subjects gave informed consent before the examinations.

**Analysis of cell-surface thiols by flow cytometry**

THP-1 cells and monocytes were seeded at 1 x 10^6 cells/ml. After treatment with test chemicals, THP-1 cells were recovered and washed with PBS twice, then incubated with 100 µl of AFM (10 µM) PBS solution for 30 min at 37°C. After having been washed again with PBS, the cells were analyzed by flow cytometry. Flow-cytometric analyses were performed with an EPICS XL-MCL System II (Beckman Coulter, Fullerton, CA, USA) or FAC-Scalibur (Becton Dickinson). Dead cells were gated out by staining with propidium iodide (PI, 0.625 µg/ml). A total of 10,000 living cells was analyzed. RFI was calculated by use of the following formula: RFI (% of control) = (MFI of chemical-treated cells/ MFI of vehicle control

**MATERIALS AND METHODS**

**Chemicals**

DNCB, DPCP, dithiothreitol (DTT) and NAC were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) and 3-(3-cholamidopropyl) dimethylammonio-1-propanesulphonate (CHAPS) were purchased from Kanto Chemical (Tokyo, Japan). Impermeable, thiol-reactive Alexa Fluor 488 C5 maleimide (AFM) and BSA Alexa Fluor 488 conjugate (AFM-BSA) were purchased from Invitrogen Life Technologies (Carlsbad, 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 the addition of fresh medium twice a week and cell density was maintained between 0.1 and 0.5 x 10^6 cells/ml.

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Quantification of intracellular GSH and GSSG

After treatment with chemicals, 0.5 x 10^6 THP-1 cells were collected by pipetting and washed twice with cold PBS. The cells were immediately lysed with 100 µl of lysis buffer (0.1% Triton X-100, 0.1 M sodium phosphate buffer, and 5 mM EDTA, pH 7.5). The mixture was allowed to stand at room temperature (RT) for 5 min to lyse the cells. Thereafter, 5 µl of 0.1 N HCl and 5 µl sulfosalicylic acid (Wako Pure Chemicals, Osaka, Japan) were added. The total cellular GSH concentration was determined using a Total Glutathione Quantification Kit (Dojindo Molecular Technologies, Gaithersburg, MD, USA). The GSSG concentration was determined according to the method of Sacchetta et al. (1986). Briefly, 1 µl of 2-vinylpyridine (Wako Pure Chemicals) was added to 50 ml of the cell lysate supernatant, and mixed at RT for 1 min, then the pH was adjusted to 7.5. Subsequently, the reaction mixture was allowed to stand at RT for 60 min. The levels of total GSH and GSSG were calculated by using a standard curve obtained with GSH and GSSG (Wako Pure Chemicals), and the content of GSH was obtained by subtracting the amount of GSSG from total GSH content.

Preparation of AFM-labeled cell membrane proteins derived from DPCP-treated THP-1

THP-1 cells were seeded at 1 x 10^6 cells/ml. After 2 hr treatment with DPCP, THP-1 cells were recovered, washed with PBS twice, and incubated with 500 µl of AFM (30 µM) PBS solution for 30 min at 37°C. The cells were washed again with PBS, and cell membrane proteins were extracted according to the protocol of 2-D Sample Prep for Membrane Proteins (Pierce, Milwaukee, WI, USA). The change of cell-surface thiols was checked by flow-cytometric analyses using the EPICS XL-MCL System II (Beckman Coulter) before the preparation of cell membrane proteins.

Analysis of AFM-labeled cell membrane proteins by 2DE

The membrane protein preparation (1 mg) was mixed in 2DE sample buffer (7 M urea, 2 M thiourea, 4% (v/v) CAPS, 60 mM DTT, 0.5% IPG buffer (pH 3-10) (GE Healthcare, Buckinghamshire, UK) and 0.002% BPB) and applied to the acidic end of immobilized pH gradient gels (Immobiline DryStrip, pH 3-10, 24 cm, GE Healthcare) in a strip holder. Protein concentration was determined using Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). Isoelectric focusing (IEF) was performed with stepwise voltage increment from 500 V to 8,000 V up to a total of 41 kV-h, using an Etan IPGphor II (GE Healthcare). Before carrying out the second-dimensional SDS-PAGE, strip gels were subjected to a two-step equilibration in equilibration buffer including 50 mM Tris-HCl (pH 8.8), 6 M urea, 30% glycerol, 2% SDS and 1% DTT and 2.5% iodoacetamide (IAA). After equilibration, gel strips were transferred onto 12.5% SDS-polyacrylamide gels (20 cm x 26 cm) and the second-dimensional SDS-PAGE was performed using Etan DALTsix Large Electrophoresis Systems (GE Healthcare). AFM-BSA was used for fluorescence correction among gels. Images of gels were digitally scanned with a FluorImager 595 (GE Healthcare) to visualize the AFM-labeled protein spots on gels. After scanning, the gels were stained using Deep Purple Total Protein stain (GE Healthcare) to visualize protein spots and scanned again. Profiles of AFM-labeled proteins and Deep Purple-stained proteins were assessed using ImageMaster 2D Platinum (GE Healthcare).

In-gel tryptic-digestion of protein spots

Protein spots of interest were manually excised from gels with a clean scalpel. The gel pieces were rinsed in deionized water, dehydrated in acetonitrile and then dried for 10 min at RT. Following reduction in 10 mM DTT and alkylation in 25 mM IAA in 25 mM ammonium bicarbonate, the gel pieces were dehydrated in acetonitrile and dried again. They were then rehydrated in 20 ng/ml of TPCK-modified trypsin (Promega, Madison, WI, USA) at 4°C. In-gel tryptic digestion was performed at 37°C overnight and digested peptides were recovered into extraction solution (2% acetonitrile, 5% formic acid). The extract was vacuum-dried and the residue was dissolved in 2% acetonitrile, 5% formic acid.

Identification of proteins using liquid chromatography coupled with electrospray tandem mass spectrometry (LC/MS/MS) analysis

Digested peptides were subjected to LC/MS/MS-based protein identification analysis as described previously (Motoyama et al., 2007). Briefly, peptide extracts were loaded by an autosampler (SI-2 semi-micro HPLC system, Shiseido Co., Ltd., Tokyo, Japan) onto a fused-silica trapping column (100 µm i.d. x 1 cm, Aqua C18, Phenomenex, Torrance, CA, USA). The trapping column was desalted with a gradient starting buffer (0.1% formic acid, 5% acetonitrile/purified water) for approximately 30 min, then it was directly connected to a fused-silica analytical capillary column (100 µm i.d. x 12 cm, Aqua C18, Phenomenex) by changing the position of a two-way switching valve. The peptides were separated with a 40-min organic gradi-
ent (5-75% acetonitrile). The column flow rate was set to 300–400 nl/min by adjusting the length of a split resistant capillary (50 μm i.d. x 50–200 mm). Peptides eluted from the column were directly electrosprayed into the mass spectrometer (Deca XP, Thermo Fisher Scientific, Waltham, MA, USA), and MS/MS spectra were automatically acquired under the control of the Xcalibur data system (Thermo Fisher Scientific). Collected MS/MS spectra were searched to identify peptides/proteins with the SEQUEST algorithm running on Bioworks software (Thermo Fisher Scientific). A non-redundant human protein database (NCBI, downloaded in 2007) was used for protein identification. Stringent search criteria were used to minimize false-discovery rates (Sf score > 0.85, Peptide probability > 0.001, Number of top matches: > 1).

**Real-time polymerase chain reaction (real-time PCR) analysis of spliced XBP1 mRNA**

Following 2-h DPCP treatment, real-time PCR analysis of spliced XBP1 mRNA was performed as described previously (Hirota et al., 2006). In brief, cDNA synthesis and measurement of spliced XBP1 mRNA were performed using a GeneAmp RNA PCR kit (Applied Biosystems, Foster City, CA, USA) and Platinum® SYBR® Green qPCR SuperMix UDG (Invitrogen Life Technologies). The double-stranded cDNA was synthesized from single-stranded cDNA and digested with PsII for 1 hr. The gene-specific PCR products were measured continuously with an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). The PCR conditions were 95°C for 5 min, then 40 cycles of 95°C 30 sec -55°C 30 sec -72°C 30 sec. The quantity of specific mRNA was normalized as a ratio to the amount of GAPDH mRNA. Specific primer sequences for real-time PCR were as follows: XBP1 mRNA, <sense primer> 5'-CCTTGTAGTTGAAGACGG-3', <anti-sense primer> 5'-GGGCTTGGTTATATATGTC-3'; GAPDH mRNA, <sense primer> 5'-GAAGTTGAGGTCCAGTC-3' <anti-sense primer> 5'-GAAGATGTTGATGGGCATTTC-3'. The values of fold increase over the control were calculated by use of the following formula: spliced XBP1 fold increase (% of control) = (normalized spliced XBP1 expression of chemical-treated cells/ normalized spliced XBP1 expression of vehicle control cells) x 100.

**SDS-PAGE and analysis of phospho-p38 MAPK and SAPK/JNK by Western blot analysis**

Following 2-h DPCP treatment, phosphorylation of JNK and p38 MAPK was determined by Western blot analysis, as described previously (Hirota et al., 2009). Immunoblotting of phosphorylated p38 MAPK and phosphorylated JNK was performed using a p38 MAPK immunoblotting kit and SAPK/JNK immunoblotting kit (Cell Signaling Technology, Beverly, MA, USA), respectively. Total cell lysate from chemical-treated cells was prepared using lysis buffer (1% Nonidet P-40, 20 mM Tris-HCl (pH 8.0), 137 μM NaCl, 10% glycerol, 2 mM ethylenediaminetetraacetic acid, 1% protease inhibitor cocktail (Sigma-Aldrich), and 1 mM sodium orthovanadate), and 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 (BPB)). The protein samples were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membranes were incubated with Block Ace (Dainippon Sumitomo Pharma, Osaka, Japan) for 60 min at RT, and then with rabbit polyclonal antibodies to anti-phosphorylated p38 MAPK, anti-p38 MAPK, anti-phosphorylated SAPK/JNK and anti-SAPK/JNK 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.

**Statistical analysis**

The statistical significance of differences in the RFI of cell surface thiols and in spliced XBP1 expression between non-treated THP-1 and chemical-treated THP-1 cells was examined using the paired Student’s t test (Snedecor and Cochran, 1989). The Bonferroni correction was applied for multiple comparisons (Wallenstein et al., 1980).

**RESULTS AND DISCUSSION**

In our previous report, we showed that modification of cell-surface thiols induces CD86 expression in cells of the human monocytic cell line THP-1, and we confirmed that 32 of 36 skin sensitizers induced a decrease of cell-surface thiols in THP-1 cells (Hirota et al., 2009; Suzuki et al., 2009). However, a few haptens, such as DPCP, MnCl2, and pyridine, caused an increase (Suzuki et al., 2009). In this study, we focused on the increase of cell-surface thiols in DPCP-treated THP-1 cells. First, we measured cell-surface thiols in DNB-C-, DPCP-, DTT- or NAC-treated THP-1 cells. Thiol antioxidants, including DTT and NAC, were reported to induce increase of cell-surface thiols (Laragione et al., 2003). As shown in Fig. 1 (A), cell-surf-
face thiols were increased by DPCP, DTT and NAC, but decreased by DNCB. The increase of cell-surface thiols by DPCP was confirmed not only in THP-1 cells, but also in primary monocytes (Fig. 1 (B)). Next, we investigated whether the intracellular GSH/GSSG ratio was affected by DPCP in THP-1 cells. It was reported that the intracellular GSH/GSSG ratio in THP-1 cells was decreased by haptens such as DNCB and NiCl₂, which induce decrease

Fig. 1. Effect of chemicals on cell-surface thiols. THP-1 cells and primary monocytes (1 x 10⁶ cells/ml) were exposed to the indicated chemicals for 2 hr. Cell-surface thiols was measured by flow cytometry as described in Materials and Methods. (A) Changes of cell-surface thiols on THP-1 cells treated with the indicated concentrations of DPCP, DNCB, DTT or NAC. Each value of RFI is the mean ± S.D. of at three independent experiments. Asterisks indicate a significant (p < 0.05) difference between chemical-treated cells and vehicle-treated cells. (B) Changes of cell-surface thiols on primary monocytes following treatment with DPCP. Representative results from three independent sets of experiments are shown.

Fig. 2. Effect of chemicals on intracellular GSH/GSSG ratio in THP-1 cells. THP-1 cells (1 x 10⁶ cells/ml) were exposed to DPCP, DNCB or DTT for 2 hr. Intracellular GSH and GSSG were measured by using colorimetric assays employing the GSH reductase-DTNB recycling procedure. The relative GSH/GSSG ratio was calculated as described in Materials and Methods. Each value of relative GSH/GSSG ratio is the mean ± S.D. of at three independent experiments. Asterisks indicate a significant (p < 0.05) difference between chemical-treated cells and vehicle-treated cells.

DPCP affects cell-surface thiols and activation in THP-1 cells
of cell-surface thiols (Mizuishi et al., 2005; Hirota et al., 2009). As shown in Fig. 2, the GSH/GSSG ratio did not change in response to treatment with DPCP or DTT. On the other hand, DNCB induced a significant decrease of the GSH/GSSG ratio. These data indicated that increase of cell-surface thiols might not be related to intracellular redox imbalance. So, we attempted to identify cell membrane proteins whose thiols were increased by means of a redox proteomics approach.

For 2DE, we used AFM, which is a membrane-impermeable thiol-reactive reagent, together with flow cytometric analysis.

Fig. 3 shows the SH group content and total protein expression of cell membrane proteins derived from cells exposed to 0.2% DMSO (solvent control, SH groups; Fig. 3A, total proteins; Fig. 3C) and 38 µM DPCP (SH groups; Fig. 3B, total proteins; Fig. 3D).

We identified twenty protein spots whose thiols were significantly augmented by DPCP treatment (Fig. 3E). At a > 2 arbitrary fold difference cut-off, thiols of twelve proteins were augmented by DPCP treatment. Among them, actin and β-tubulin are cytoskeletal components. It was reported that the thiol content of β-actin was increased by DPCP (38 µM) DMSO (0.2 %)
NAC treatment in human PBMC (Laragione et al., 2003). Adenylyl cyclase-associated protein (CAP) was reported to bind actin (Hubberstey et al., 1996). Cofilin and Hsp 27 were reported to be important in the control of the actin cytoskeletal network (de Graauw et al., 2005). Furthermore, cofilin is reported to translocate to plasma membranes, and to be involved in actin depolymerization, cell motility, and apoptosis in human prostate cancer cells (Suzuki et al., 1995; Zhu et al., 2006). Prohibitin is located in plasma membrane and mitochondria, and was reported to be responsive to apoptosis signaling by TGF-β (Mielenz et al., 2005; Zhu et al., 2006). Annexin IV was also reported to be located in plasma membrane, and to interact with activated protein kinase C alpha (Diakonova et al., 1997; Schmitz-Peiffer et al., 1998). These data supported the idea that conformational changes of cell membrane proteins, such as actin and β-tubulin, may result in apoptotic signaling.

Rab1B, belonging to the Ras superfamily of GTPases, is required for protein transport from ER to Golgi (Alvarez et al., 2003). Glyoxalase I is related to the detoxification of α-oxoaldehyde and its expression is augmented in Parkinson’s disease, possibly via endoplasmic reticulum (ER) stress (de Hemptinne et al., 2007; Ryu et al., 2002; Werner et al., 2008).

From the 2DE data, we identified molecular chaperones (Hsp 27 and Hsp 70) and ER stress-related proteins (palmitoyl-protein thioesterase 1 (PPT1), stromal-cell derived factor 2-like 1 (SDF2L1)). Hsp 27, which was identified from two spots with the same molecular weight and different PI, might have been phosphorylated. PPT is related to the post-translational modification of proteins and PPT gene mutation was reported to cause ER stress (Kim et al., 2006). SDF2L1 was also reported to be induced by ER stress (Fukuda et al., 2001). These data may indicate that increase of cell-surface thiols is associated with ER stress.

In general, heat shock proteins, such as Hsp 27 and Hsp 70 act as molecular chaperones, promoting the folding of proteins. Protein folding is performed in the ER, and the aggregation of unfolded or misfolded proteins leads to apoptosis via ER stress (Rao et al., 2004). Thiols in proteins are known to play a crucial role in protein folding. Cell-surface proteins include thiol-containing ion channels and membrane receptors that are redox-sensitive (Garant et al., 1999; Lipton et al., 2002; Zeng et al., 2003). Based on our data and previous reports, we speculated that chemically induced increase of thiols may result in alterations of protein structure that lead to ER stress. Therefore, we examined the increase of spliced XBP1 mRNA expression, a biomarker of ER stress, and the phosphorylation of intracellular SAPK/JNK and p38 MAPK in THP-1 cells. THP-1 cells were exposed to test chemicals for 2 hr and cell lysate was prepared as described in Materials and Methods. Western blot analysis of phosphorylated SAPK/JNK and p38 MAPK was performed using p38 MAPK immunoblotting kit and SAPK/JNK immunoblotting kit, respectively. Representative results from three independent sets of experiments are shown.
ylation of p38 MAPK and JNK/SAPK (Figs. 4 and 5). In Fig. 4, we used 113 µM DPCP as well as 38 µM DPCP in order to examine the dose-dependence of the expression of spliced XBP1 mRNA. XBP1 mRNA was reported to be spliced by activated IRE1α, a sensor protein of ER stress (Yoshida et al., 2001). IRE1α was reported to activate apoptosis signal-regulating kinase 1 (ASK1) and its downstream signal transduction molecules, such as p38 MAPK and c-Jun NH₂-terminal kinases (JNK, including JNK1, JNK2 and JNK3 isoforms) (Nagai et al., 2007). These data are consistent with the activation of MAPK pathways via IRE1α by DPCP. Among cell-surface thiol-increasing reagents, DTT provoked ER stress, including JNK activation (Urano et al., 2000), while MnCl₂ induced ER stress, including augmentation of BiP and activation of caspase-12 (Chun et al., 2001).

Based on the above results, we hypothesized that conformational change of cytoskeletal proteins might be induced by way of intracellular signaling molecules such as HSP27 and cofilin in DPCP-treated THP-1 cells. Furthermore, increase of thiols in cell-surface proteins might lead to activation of signal transduction in the MAPK pathway via IRE1α, resulting in ER stress.

However, our results did not establish the mechanism of the increase of cell-surface thiols. For example, protein disulfide isomerase (PDI) was not hit in the redox proteomics analysis, though it was reported to control membrane thiols, and might be a target of DPCP (Jiang et al., 2007). Further work is needed to investigate the effects of chemicals on key molecules which control the redox state of cell membrane proteins.

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