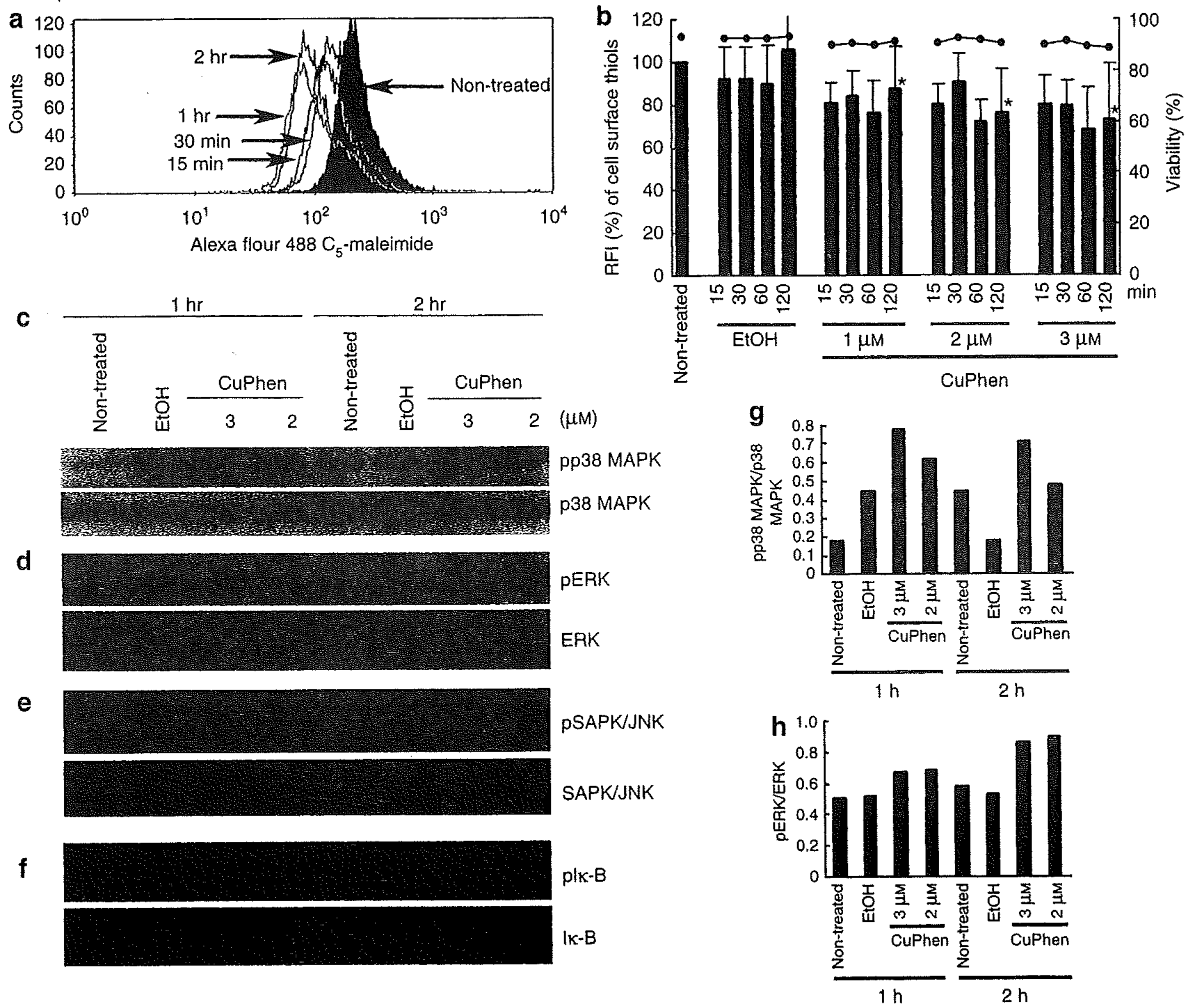

Figure 2. An impermeant thiol oxidizer, CuPhen, decreases cell surface thiols of MoDCs and phosphorylates p38 MAPK. MoDCs were treated with three different concentrations of CuPhen for different time periods. The cell surface thiols were quantified by flow cytometry using thiol-reactive Alex Fluor 488 C5 maleimide. A representative flow cytometry of MoDC treated with 3 μM CuPhen (**a**) and the summarized data from five different experiments (**b**) are presented. The mean ± SEM of RFI is shown by black bars. A line chart shows cell viability. Asterisks indicate significance (**P*<0.05) for the difference between stimulated cells and the vehicle-treated control. After 1 or 2 hours of treatment with CuPhen, phosphorylation of p38 MAPK was examined by western blotting using anti-pp38 MAPK antibody (**c**), anti-phospho-p42/44 ERKs antibody (**d**), phospho-SAPK/JNK antibody (**e**), or anti-serine (Ser32/36) phosphorylated IκB antibody (**f**). As a control to confirm that equal amounts of protein were loaded to the gel, p38 MAPK, p42/44 ERKs, SAPK/JNK, and IκB expression are also shown. To show the relative ratio of phosphorylated p38 MAPK *versus* total p38 MAPK (**g**) or phosphorylated p42/44 ERKs *versus* total p42/44 ERKs (**h**), the optical densities of the signals were measured with NIH image. These are representative data from two to four different experiments. RFI, relative fluorescence intensity.

whether sensitizers decreased cell surface thiols. The sensitizers that we examined, that is, DNCB, NiSO4, formalin, dephencyprone, and squaric acid dibutyl ester, significantly decreased the cell surface thiols of MoDCs, whereas a representative irritant, SDS, or a danger signal, ATP, did not affect them at all.

Given that sensitizers can penetrate into the cytoplasm and affect the intracellular redox balance, it is not clear whether the oxidation of cell surface thiols of MoDCs by sensitizers induces p38 MAPK and DC maturation. Therefore, we next examined whether an impermeant thiol oxidizer, CuPhen (Duncan and Lawrence, 1989; Lawrence *et al.*, 1996), can phosphorylate p38 MAPK of MoDCs and stimulate DC maturation. After we confirmed that CuPhen decreased cell surface thiols of MoDCs, we showed that CuPhen phosphorylated p38 MAPK and induced DC maturation, that is, the augmentation of HLA-DR, CD83, and CD86 expression, the induction of IL-8 mRNA, and the downregulation of AQP3 mRNA. These data suggest that the oxidation of cell surface thiols by sensitizers can trigger the maturation of MoDCs.

In our previous paper, we reported that NAC recovered the decreased intracellular GSH/GSSG ratio induced by sensitizers and, concomitantly, abrogated the phosphorylation of p38 MAPK and the augmentation of CD86 expression by MoDCs. Recently, it was reported that some effects of NAC, for example, inhibition of apoptosis, antiproliferative effect, inhibition of epidermal growth factor receptor activation, or enhancement of IL-1-induced inducible nitric oxide synthase expression, seemed not to be mediated by an increase of

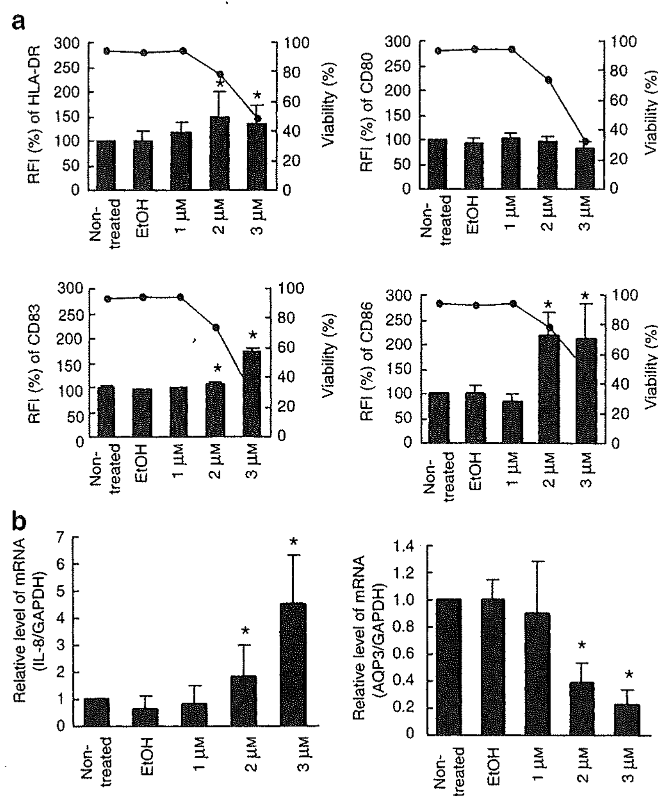

Figure 3. CuPhen induces the maturation of MoDCs. (**a**) After 24 hours treatment with three different concentrations of CuPhen, the expression of HLA-DR, CD80, CD83, and CD86 antigen by MoDCs was examined by flow cytometry. Summarized data from five different experiments are shown. The mean ± SEM of RFI is shown by black bars. A line chart shows cell viability. Asterisks indicate significance (*$P<0.05$) for the difference between stimulated cells and the vehicle-treated control. (**b**) After 6 hours of treatment, IL-8 and aquaporin-3 mRNA expressions were examined by real-time PCR. Summarized data from three different experiments are shown. The mean ± SEM of the relative level of IL-8 mRNA and aquaporin-3 mRNA is shown. Asterisks indicate significance (*$P<0.05$) for the difference between stimulated cells and the vehicle-treated control. RFI, relative fluorescence intensity.

GSH, as suggested by experiments with inhibitors of GSH synthesis or those with the D-stereoisomer of NAC (D-NAC) that cannot be converted to GSH (reviewed by Laragione *et al.* (2003)). Indeed, in this study, we showed that NAC significantly increased the cell surface thiols of MoDCs, and suppressed the phosphorylation of p38 MAPK and MoDC maturation induced by CuPhen. These data suggest that the inhibitory effects of NAC on MoDC maturation by sensitizers are at least partially mediated by its thiol-disulfide exchange activity as a reductant.

The cytoplasm represents a reducing environment, where most of the protein cysteines are in a reduced state owing to the high level of intracellular GSH (Hwang *et al.*, 1992); the extracellular environment is highly oxidizing and most known secreted proteins are very rich in disulfides (Thornton, 1981). Proteins on the cell membrane are at the interface between these two compartments and may have a key role as sensors of the environmental redox state, as their location makes them particularly sensitive to exogenous oxidants or reductants (Laragione *et al.*, 2006). Our current study showing that sensitizers oxidize cell surface thiols, causing the phosphorylation of p38 MAPK and activation of MoDCs, which are suppressed by a p38 MAPK inhibitor, seems to support this notion; that is, DCs sense sensitizers through the oxidation of cell surface thiols and transduce the signal into the phosphorylation of p38 MAPK that is well known to have a crucial role in DC maturation. Indeed, when we applied DNCB to the mouse skin, LC reduced their cell surface thiols, which suggests that sensitizers trigger LC maturation through the oxidation of cell surface thiols.

Interestingly, CuPhen also phosphorylated p42/44 ERKs. Recently, the concomitant phosphorylation of both p38 MAPK and p42/44 ERKs by reactive oxygen species have been shown through the analysis of the effects of cigarette smoke extract on DCs (Kroening *et al.*, 2008). Therefore, our study suggests that the oxidation of cell surface thiols can phosphorylate both p38 MAPK and p42/44 ERKs. In contrast to the positive role of p38 MAPK in DC maturation, which has been confirmed by a recent elegant study using a dominant active form of MAPK kinase 6, a direct upstream kinase of p38 MAPK (Jorgl *et al.*, 2007), several studies suggested the negative role of p42/44 ERKs in DC maturation, such as the upregulation of IL-12p40 and CCR7 (Li *et al.*, 2007; Kroening *et al.*, 2008). We have also reported that the suppression of p42/44 ERKs by PB98059 rather augmented CD86 expression by sensitizer-stimulated MoDCs, whereas the suppression of p38 MAPK by SB203580 significantly suppressed it (Aiba *et al.*, 2000). These studies suggest the negative role of p42/44 ERKs in DC maturation. Therefore, the role of the phosphorylation of p42/44 ERKs on CuPhen-induced DC maturation remains to be determined.

On the other hand, in contrast to several papers (Arrighi *et al.*, 2001; Aiba *et al.*, 2003; Boisleve *et al.*, 2004) showing the phosphorylation of SAPK/JNK by haptens, CuPhen did not phosphorylate SAPK/JNK. Recently, Handley *et al.* (2005) have reported that hydrogen peroxide at low concentrations phosphorylates p38 MAPK more strongly than SAPK/JNK, whereas it phosphorylates both p38 MAPK and SAPK/JNK in the same magnitude at higher concentrations. These data suggest that there is a hierarchic activation of SAPK/JNK and p38 MAPK, in which incremental reactive oxygen species stimulation sequentially activates p38 MAPK and SAPK/JNK. The lack of SAPK/JNK phosphorylation by CuPhen-treated MoDCs suggests that CuPhen did not activate MoDCs to a level sufficient to stimulate SAPK/JNK. In addition, Thoren *et al.* (2007) have shown that MoDCs have a unique capacity to neutralize extracellular oxygen radials and express higher levels of cell surface thiols than monocytes, which suggests that MoDCs are less sensitive to extracellular oxidative stress.

Finally, as many previously unknown chemicals that are likely to enter into contact with the skin are synthesized every day, it is essential to be able to predict their sensitizing potential. Accordingly, one of the goals of the research on the mechanism of allergic contact dermatitis is to develop non-animal test methods for skin sensitization testing. Several possible methods using DCs have been reported (reviewed by Ryan *et al.* (2007)). In these *in vitro* tests,

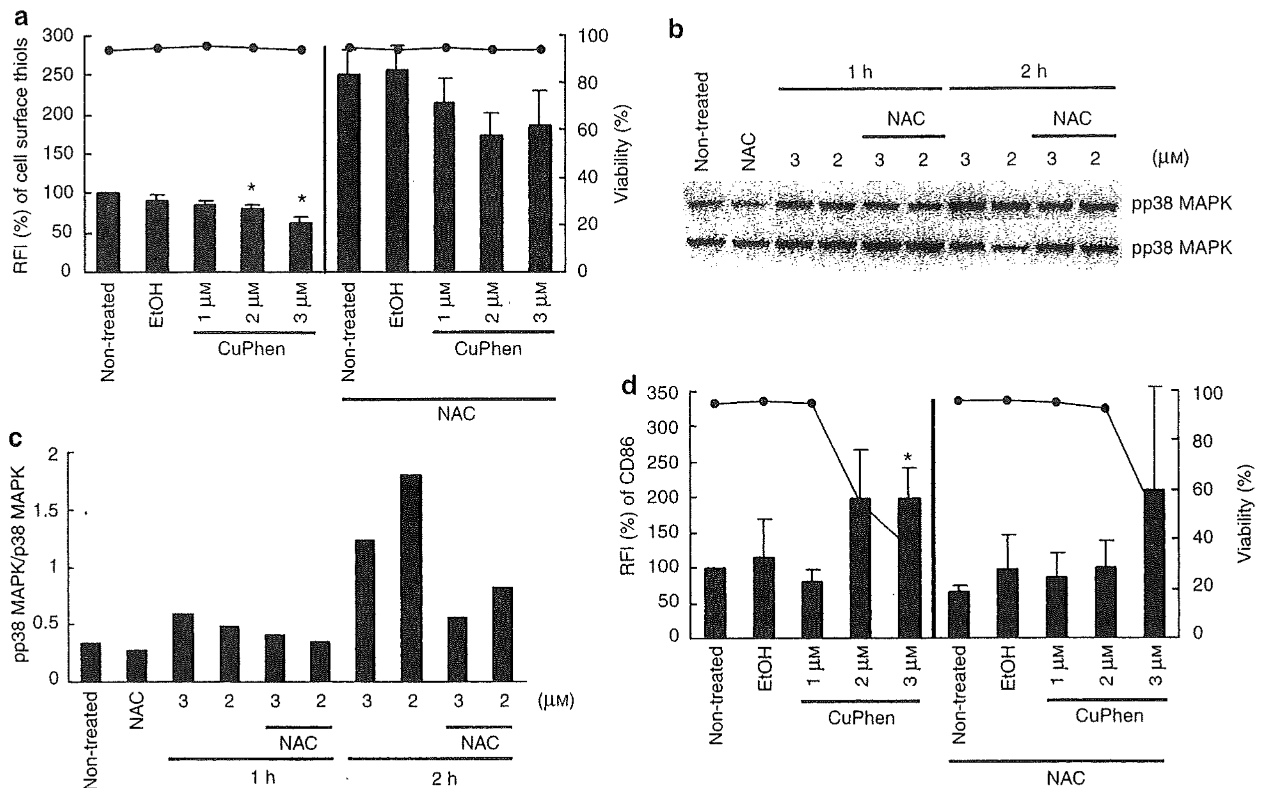

Figure 4. The pretreatment of NAC significantly increases cell surface thiols of MoDCs and suppresses the effects of CuPhen on MoDCs. To examine the effects of the antioxidant NAC, MoDCs were pretreated with 25 mM NAC for 30 minutes, washed with the culture medium, and stimulated by CuPhen. (**a**) After 2 hours treatment with CuPhen, the cell surface thiols were quantified by flow cytometry using thiol-reactive Alex Fluor 488 C5 maleimide. The mean ± SEM of RFI for cell surface thiols from three independent experiments is shown. Asterisks indicate significance (*$P<0.05$) for the difference between stimulated cells and the non-treated control. (**b**) After 1 hour or 2 hours treatment with CuPhen, the phosphorylation of p38 MAPK was examined by western blotting using anti-pp38 MAPK antibody. As a control to confirm that equal amounts of protein were loaded to the gel, total p38 MAPK is also shown. (**c**) To show the relative ratio of phosphorylated p38 MAPK *versus* total p38 MAPK, the optical densities of the signals were measured with NIH image. Shown are representative data from two different experiments. (**d**) After 24 hours treatment, the expression of CD86 antigen by MoDCs was examined by flow cytometry. Summarized data from three different experiments are shown. The mean ± SEM of RFI is shown by black bars. A line chart shows cell viability. Asterisks indicate significance (*$P<0.05$) for the difference between stimulated cells and the vehicle-treated control. RFI, relative fluorescence intensity.

the chemicals used to stimulate DCs or other cell lines are usually used at sublethal concentrations. Therefore, they may release ATP that stimulates DCs, as shown by Mizumoto *et al.* (2003). In this study, we clearly showed that ATP did not affect the cell surface thiols of MoDCs, which suggests that an assessment of cell surface thiols may be able to discriminate the stimulation by sensitizers from that by nonspecific signals of ATP. On the basis of this concept, we have developed a previously unreported *in vitro* skin sensitization test, in which we evaluate the changes of cell surface thiols on human monocyte cell line, THP-1 cells, after chemical treatment as a biomarker for skin sensitization (Suzuki *et al.*, 2009). It is clearly necessary to examine the effects of large numbers of chemicals, including haptens, prohaptens, and irritants, on the cell surface thiols of MoDCs or THP-1 to determine the possibility and limitations of this method as a non-animal test method for skin sensitizing testing.

MATERIALS AND METHODS

Media and reagents

The medium used in this study was RPMI-1640, including 25 mM Hepes buffer (Sigma-Aldrich, St Louis, MO) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 1% penicillin, streptomycin, and fungizone antibiotic solution (Sigma), and 10% fetal calf serum (Bioserum, Canterbury, Victoria, Australia) (complete medium). MACS colloidal supermagnetic microbeads conjugated with anti-human CD14 mAb (CD14 microbeads) were purchased from Miltenyi Biotec (Sunnyvale, CA). Recombinant human GM-CSF and recombinant human IL-4 were purchased from PeproTech (London, UK). The buffer used for the purification of $CD14^+$ monocytes from peripheral blood mononuclear cells was phosphate-buffered saline (PBS) supplemented with 1% BSA (less than 1 ng ml^{-1} of detectable endotoxin) (Sigma) and 5 mM EDTA (MACS buffer). Cu(II)SO_4, 1,10-phenanthroline (Sigma), DNCB, dephencyprone, $NiSO_4$, SDS (Sigma), formalin (HCHO), ATP (WAKO Pure Chemicals, Osaka, Japan), and squaric acid dibutyl ester (Tokyo Kasei Kogyo, Tokyo, Japan) were used for the stimulation of MoDCs. CuPhen was prepared by dissolving Cu(II)SO_4 and 1,10-phenanthroline (Sigma) in a 4:1 water/ethanol solution. $NiSO_4$, HCHO, SDS, and ATP were solubilized in distilled water, whereas DNCB, dephencyprone, and squaric acid dibutyl ester were solubilized inDMSO (Sigma). The final concentration of DMSO was always less than 0.1% and cultures of MoDCs with 0.1% DMSO were also examined as control. The antioxidant NAC, and p38 MAPK inhibitor

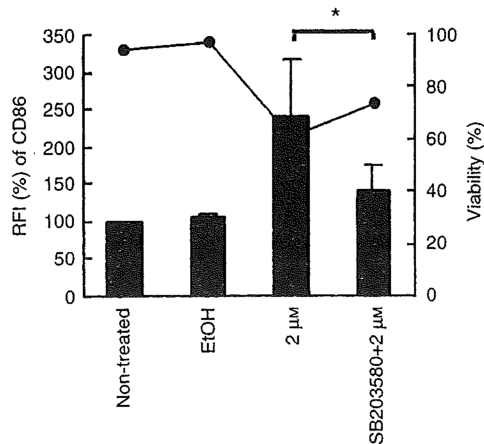

Figure 5. The treatment of SB203580 suppresses the augmentation of CD86 by CuPhen-treated MoDCs. To examine the effects of SB203580, we added 30 μM SB203580 to the culture of MoDCs 1 hour before stimulation by CuPhen and stimulated them using CuPhen. After 24 hours stimulation, the expression of CD86 antigen by MoDCs was examined by flow cytometry. Summarized data from three different experiments are shown. The mean ± SEM of RFI is shown by black bars. A line chart shows cell viability. Asterisks indicate significance (*$P<0.05$) for the difference between stimulated cells and the vehicle-treated control. RFI, relative fluorescence intensity.

SB203580 hydrochloride were purchased from Sigma and Calbiochem (San Diego, CA), respectively, and solubilized in distilled water, and then the pH was adjusted to 7.4. The endotoxin content of the final dilution used was $<30\,pg\,ml^{-1}$, as determined by the Limulus amebocyte lysate assay (Seikagaku, Tokyo, Japan).

We used the following mAbs for immunostaining: FITC-conjugated-anti-HLA-DR, FITC-conjugated-anti-CD80, phycoerythrin(PE)-conjugated-anti-CD86 (BD Pharmingen, San Diego, CA), PE-conjugated-anti-CD83 (Beckman Coulter, Fullerton, CA), FITC or PE-conjugated isotype-matched mouse control Abs (IgG1, IgG2a, and IgG2b), and PE-conjugated anti-phospho-p38 MAPK (T180/Y182) Ab (BD Pharmingen). 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.

Culture of MoDCs from peripheral blood monocytes and chemical treatment with sensitizers

Peripheral blood mononuclear cells were isolated from heparinized fresh leukocyte-enriched buffy coats from different donors using Lymphoprep (Axis-Shield PoC As, Oslo, Norway). After several washes with PBS, 1×10^8 peripheral blood mononuclear cells were treated with 200 μl of CD14 microbeads in 800 μl of MACS buffer at 4 °C for 30 minutes. After washing with MACS buffer, the cells coated with CD14 microbeads were 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 and used cell specimens containing more than 98% $CD14^+$ cells in the experiments.

$CD14^+$ monocytes (2×10^6 per ml) were cultured in complete medium containing $100\,ng\,ml^{-1}$ of recombinant human GM-CSF and $100\,ng\,ml^{-1}$ of recombinant human IL-4 for 6 days. One-half of the culture medium was changed on days 3 and 6. On the day 6, the

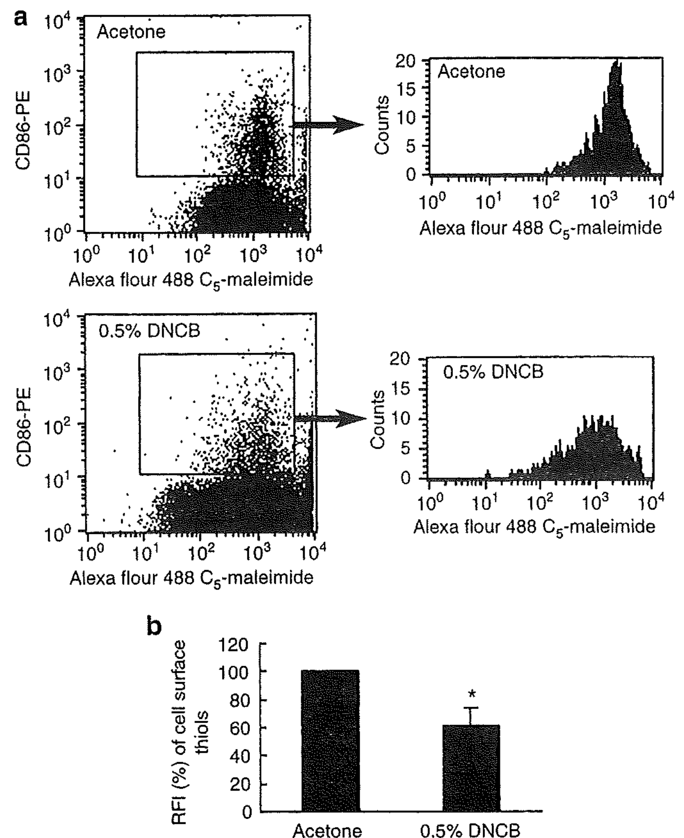

Figure 6. Epidermal langerhans cells decrease cell surface thiols after epicutaneous application of DNCB. The freshly shaved abdomens of BALB/c mice were painted with 100 μl of 0.5% of DNCB. After 2 hours, epidermal cell suspensions were prepared from the applied skin. These cell suspensions were stained with PE-conjugated anti-CD86 antibody and Alexa fluor 488 C_5 maleimide. The cell surface thiols of $CD86^+$ epidermal cells were compared between DNCB-treated mice and vehicle-treated mice. A representative flow cytometry (**a**) and the summarized data from three different experiments (**b**) are shown. The mean ± SEM of RFI is shown by black bars. Asterisk indicates significance (*$P<0.05$) for the difference between stimulated cells and the vehicle-treated control. RFI, relative fluorescence intensity.

cells were treated with different concentrations of DNCB, dephencyprone, squaric acid dibutyl ester, $NiSO_4$, HCHO, SDS, or ATP. After 2 hours, the cell surface thiols of these cells were examined.

Stimulation of MoDCs with CuPhen in the presence or absence of NAC or SB203580

Monocyte-derived DCs were treated with various concentrations of CuPhen for different time periods. In some experiments, to examine the effects of the antioxidant NAC, MoDCs were exposed to 25 mM NAC for 30 minutes, washed with the culture medium, and stimulated by the chemicals. To examine the effects of p38 MAPK inhibitor, cells were treated with 30 μM of SB203580, as we have reported previously (Aiba *et al.*, 2003).

Flow cytometry for MoDCs

To stain cell surface thiols, MoDCs were recovered from the culture, washed with PBS twice, and then incubated with 100 μl of Alexa Fluor 488 C_5 maleimide (Invitrogen) PBS solution (10 μM) for 30 minutes at 37 °C. To analyze the intracellular expression of phospho-p38

MAPK, we permeabilized the cells using Fix & Perm reagents (Caltag Laboratories, Burlingame, CA) according to the manufacturer's protocol, and cell staining was performed using a PE-conjugated anti-phospho-p38 MAPK (T 180/Y 182). For immunophenotyping, cell staining was performed using FITC-conjugated-anti-HLA-DR, FITC-conjugated-anti-CD54, FITC-conjugated-anti-CD80, PE-conjugated-anti-CD86, PE-conjugated-anti-CD83 Ab, and FITC or PE-conjugated isotype-matched mouse control Abs (10 μg ml^{-1}). After washing with FACS buffer, the cells were analyzed by FACScan using CellQuest software (Becton-Dickinson, San Jose, CA). Dead cells were gated out after staining with 0.5 mg ml^{-1} propidium iodide solution for non-permeabilized cells. In some experiments, relative fluorescence intensity (RFI) was calculated by using the following formula: RFI (%) = (Mean fluorescence intensity (MFI) of chemical-treated cells/MFI of vehicle control cells) × 100. To calculate cell viability, dead cells were counted as propidium iodide(+) cells and viability (%) was calculated by dividing the numbers of dead cells by those of propidium iodide(−) living cells ± dead cells.

Analysis of phospho-MAPKs and phospho-IκB by immunoblotting

Monocyte-derived DCs were either unstimulated or stimulated with CuPhen for 1 or 2 hours. To examine the effects of NAC or p38 MAPK inhibitor, cells were treated with 25 mM NAC or 30 μM of SB203580, as we have described above. The cells were washed twice in cold PBS and resuspended in 250 μl of lysis buffer (1% Nonidet P-40, 20 mM Tris-HCl (pH 8.0), 137 μM NaCl, 10% glycerol, 2 mM EDTA, 10 μg ml^{-1} leupeptin, 10 μg ml^{-1} aprotinin, 1 mM phenylmethanesulfonyl fluoride, and 1 mM sodium orthovanadate). The nuclei and the insoluble cell debris were removed by centrifugation at 4 °C for 10 minutes at 14,000 × *g*. The postnuclear extracts were collected and used as the total cell lysates. The total cell lysates were suspended in 2 × SDS sample buffer (313 mM Tris-HCl (pH 6.8), 10% SDS, 2-mercaptoethanol, 50% glycerol, and 0.01% bromophenol blue) and heated at 95 °C for 3 minutes. The protein samples were subjected to SDS-PAGE and transferred onto nitrocellulose membranes. The nonspecific Ab binding sites on the membranes were blocked with 1% BSA, 0.01% Tween20 in saline (10 mM Tris-HCl (pH 7.4) 100 mM NaCl) for 20 minutes at 37 °C. Immunoblotting of phosphorylated p42/44 ERKs, SAPK/JNK, p38 MAPK, or IκB was performed using p42/44 ERKs, SAPK/JNK, p38 MAPK, or IκB immunoblotting kits purchased from Cell Signaling Technology (Beverly, MA), respectively, as we have reported previously (Aiba *et al.*, 2003). Briefly, the membranes were incubated for 16 hours at 4 °C with rabbit polyclonal Abs to anti-tyrosine phosphorylated p42/44 ERKs, SAPK/JNK, p38 MAPK, or anti-serine (Ser32/36) phosphorylated IκB, washed for 15 minutes, and incubated with horseradish peroxidase-conjugated secondary Abs for 1 hour at room temperature. Blots were visualized by enhanced chemiluminescence. To ensure that there were similar amounts of MAPKs or IkB in each sample, the same membranes were stripped off, reprobed with Abs to p42/44 ERKs, SAPK/JNK, p38 MAPK, or IkB and developed with horseradish peroxidase-conjugated secondary Abs by enhanced chemiluminescence.

Quantitative real-time reverse transcription PCR

Quantitative, fluorescent PCR was performed using the TaqMan system (ABI 7700: Applied Biosystems, Foster City, CA). Sequences for human GAPDH, IL-8, and AQP3 were obtained from GenBank. We chose forward and reverse primers to span exon–intron boundaries. TaqMan probes were chosen to be used with these primers using Primer Express version. 1.0 (Applied Biosystems). Forward and reverse primers were made by Operon (Nihon Gene Research Laboratories, Sendai, Japan), whereas TaqMan probes were made by Applied Biosystems. The primers used were as follows: IL-8, forward 5′-GTGTGTAAACATGACTTCCAAGCTG-3′, reverse 5′-TCTTTAGCACTCCTTGGCAAAAC-3′; AQP3, forward 5′-CCCAT CGTGTCCCCA-3′, reverse 5′-GCCGATCATCAGCTGGTACA-3′; GAPDH, forward 5′-GAAGGTGAAGGTCGGAGTC-3′, reverse 5′-GAA GATGGTGATGGGATTTC-3′. Probes, 5′-(FAM490-labeled)-3′(tetra-methylrhodamine-labeled), were as follows: IL-8, 5′-TGATTTCTGC AGCTCTGTGTCAAGGTGC-3′; AQP3, 5′-TGATTTCTGCAGCTCTG TGTGAAGGTGC-3′; GAPDH, 5′-TGGCAAATTCCATGGGACCGT CA-3′. RNA was extracted by using the guanidinium thiocyanate method described by the manufacturer (ISOGEN; Nippon gene, Toyama, Japan) from MoDCs either unstimulated or stimulated using CuPhen for 6 hours. First strand cDNA was synthesized from total RNA extracted in RNase-free conditions. cDNA was obtained from total RNA using TaKaRa RNA PCR kit (AMV) (Takara Biochemicals, Osaka, Japan), as described by the manufacturer's protocol. PCR for GAPDH, IL-8, and AQP3 were performed in triplicate in 30 μl total reaction volumes using 66 nM TaqMan probe, 400 nM forward primers, 400 nM reverse primers, and 2 × TaqMan universal PCR Master (Applied Biosystems). Thermal cycling was performed for 2 minutes at 50 °C for depleting contaminated RNA, 10 minutes denaturation at 95 °C, followed by 40 cycles at 95 °C for 15 seconds, and at 60 °C for 1 minute in the ABI Prism 7700 detection system (Applied Biosystems). The levels of cDNA for GAPDH, IL-8, and AQP3 generated from cellular RNA were calculated by using standard curves generated with bona fide human cDNAs for GAPDH, IL-8, or AQP3, in which there was linear relationship between the number of cycles required to exceed the threshold and the number of copies of cDNA added.

Mice

BALB/c mice were obtained from Japan SLC (Shizuoka, Japan). Female mice, 8 weeks old, were used. The study was approved by the Animal Research Committee of Tohoku University and the ethics committee of Shiseido Research Center in accordance with the National Research Council Guidelines and the National Institute of Health.

Preparation of murine epidermal cell suspensions

In total 100 μl of 0.5% DNCB in acetone:olive oil (4:1) was applied to the freshly shaved abdomen of mice. Control mice were painted on the shaved abdomen with 100 μl of the vehicle. After 2 hours, skin sheets from abdomens were floated in 0.1% trypsin in PBS (pH 7.4) for 30 minutes at 37 °C. The epidermis was peeled in 0.1% DNase I solution (0.05% DNase I in PBS supplemented with 25% fetal calf serum), injected vigorously, and filtered through nylon mesh.

Flow cytometry of murine epidermal cell suspensions

Murine epidermal cells were washed with FACS buffer and then incubated with 100 μl of Alexa fluor 488 C_5 maleimide PBS solution (10 μM) for 30 minutes at 37 °C. After washing with FACS buffer, the cells were stained using a PE-conjugated anti-mouse CD86 ab. After washing with FACS buffer, the cells were analyzed by FACScalibur

using CellQuest software (Becton-Dickinson). Viable cells were identified by 7-AAD (BD Pharmingen) uptake.

Statistical analysis

Data were presented as mean ± SEM, and the statistical significance was analyzed using paired Student's *t*-test and considered to be significant at $P<0.05$. To examine the correlation between the cell surface thiol expression and phosphorylation of p38 MAPK, we calculated all the data from MoDCs stimulated with various concentrations of chemicals by Pearson's correlation coefficients.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

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