Redox Imbalance Induced by Contact Sensitizers Triggers the Maturation of Dendritic Cells

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Although p38 mitogen-activated protein kinases (MAPK) play a crucial role in the activation of monocyte-derived dendritic cells (MoDC) by contact sensitizers, the upstream signals of p38 MAPK remain undetermined. To examine whether sensitizers induce redox or oxidative stress in dendritic cells (DC), which subsequently stimulate p38 MAPK, we measured the ratio of the oxidized (GSSG) *versus* reduced (GSH) form of cellular glutathione in MoDC stimulated with five sensitizers including $NiCl_2$ and 2,4-dinitrochlorobenzene (DNCB) and three non-sensitizers including sodium dodecyl sulfate using colorimetric assays. All the sensitizers, but none of the non-sensitizers at sublethal concentration, reduced the GSH/GSSG ratio, which was accompanied by phosphorylation of p38 MAPK. Treatment with the antioxidant, *N*-acetyl-L-cysteine, which suppressed the reduction of the GSH/GSSG ratio, abrogated both the phosphorylation of p38 MAPK and the augmentation of CD86 expression. A similar response pattern was observed in THP-1 macrophage-monocyte cells. Unexpectedly, however, formaldehyde (HCHO) reduced the GSH/GSSG ratio in MoDC, but not in THP-1. This finding, in conjunction with the observation that DNCB and $NiCl_2$ reduced the GSH/GSSG ratio at different kinetics, indicated that the sensitizers reduced the GSH/GSSG ratio by a different mechanism. These data suggest that the GSH/GSSG imbalance plays a crucial role in triggering DC maturation by sensitizers.

Key words: dendritic cell/glutathione/p38 MAPK/redox/sensitizer
J Invest Dermatol 124:579–586, 2005

We reported that murine Langerhans cells (LC) upregulate their expression of class II MHC antigen and several costimulatory 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). In addition to these *in vivo* studies, using human monocyte-derived dendritic cells (MoDC) or dendritic cells (DC) derived from $CD34^+$ hematopoietic progenitor cells (HPC), we (Aiba *et al*, 1997) and others (Coutant *et al*, 1999; Arrighi *et al*, 2001; De Smedt *et al*, 2001) demonstrated *in vitro* that purified DC respond to sensitizers such as $NiCl_2$ and 2,4-dinitrochlorobenzene (DNCB), but not to irritants such as benzalkonium chloride (BC) or sodium dodecyl sulfate (SDS) by significantly augmenting their expression of CD54, CD86, and HLA-DR and by increasing their production of pro-inflammatory cytokines. Furthermore, using LC-like DC induced in the presence of transforming growth factor β1 (Geissmann *et al*, 1998), we have shown that *in vitro* treatment with sensitizers can induce phenotypic and functional changes in these DC as seen in epidermal LC during the initiation phase of contact hypersensitivity reaction *in vivo*, e.g., the expression of CCR7 that enable LC to respond to MIP-3β (Aiba *et al*, 2000). Recently, Boisleve *et al* (2004) have also reported that nickel and DNCB induce CCR7 expression on human DC derived from $CD34^+$ HPC.

In spite of these observations, however, it is still unknown how sensitizers with different chemical structures can stimulate MoDC to acquire the mature phenotype. Recently, using MoDC, Arrighi *et al* (2001), Brand *et al* (2002) and Aiba *et al* (2003) have reported that several sensitizers, i.e., 2,4-dinitrofluorobenzene, DNCB, and $NiSO_4$, induce p38 mitogen-activated protein kinase (MAPK) and/or extracellular signal-regulated kinases (ERK) phosphorylation, and that the phenotypic and functional changes induced by these sensitizers are suppressed by inhibitors of p38 MAPK or ERK. So, the next question is how these MAP kinases are activated by sensitizers in spite of the lack of proved specific receptors.

In this study, we focused on the mechanism of p38 MAPK activation by sensitizers. In general, the oxidized/reduced glutathione (GSSG/2GSH) couple provides a very large pool of reducing equivalents and is considered to be a cellular redox buffer (Schafer and Buettner, 2001). Recently, it has been demonstrated that a cellular redox imbalance could be induced by reactive oxygen species (ROS) or by thiol oxidants such as diamide without generating ROS (Pias and Aw, 2002) and that the redox imbalance detected as changes in the intracellular GSH/GSSG ratio modulates

Abbreviations: BC, benzalkonium chloride; DC, dendritic cell; DMSO, dimethyl sulfoxide; DNCB, 2,4-dinitrochlorobenzene; ERK, extracellular signal-regulated kinases; GSH, reduced glutathione; GSSG, oxidized glutathione; HPC, hematopoietic progenitor cell; LC, Langerhans cell; MAPK, mitogen-activated protein kinase; MoDC, monocyte-derived dendritic cell; NAC, *N*-acetyl-L-cysteine; PI, propidium iodide; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate

MAPK and various transcription factors related to growth, differentiation, and death (Nordberg *et al*, 1998; Schafer and Buettner, 2001). Among MAPK, p38 MAPK has been demonstrated to be phosphorylated by oxidizing conditions via apoptosis signal-regulating kinase 1 modulation (Saitoh *et al*, 1998).

Therefore, in this study, we examined the relationship between the GSH/GSSG ratio and phosphorylation of p38 MAPK in human MoDC stimulated with various sensitizers having different chemical structures. We found that all the sensitizers at a sublethal concentration reduced the GSH/GSSG ratio in MoDC, and that this was accompanied by the phosphorylation of p38 MAPK. In some experiments, we used an antioxidant, *N*-acetyl-L-cysteine (NAC), which increases the intracellular concentration of GSH (Zafarullah *et al*, 2003), to further investigate the relationship between the redox and the activation of p38 MAPK and that between the redox and the activation of MoDC, such as the augmentation of CD86 expression. The treatment with NAC, which suppressed the reduction of the GSH/GSSG ratio in MoDC, abrogated both the phosphorylation of p38 MAPK and the augmentation of CD86 expression by MoDC. These data support the role of the redox imbalance in stimulating DC maturation by sensitizers.

Results

Some contact sensitizers at the sublethal concentration upregulated the expression of CD86 in MoDC In this study, we focused on the cellular redox state and the activation of p38 MAPK, both of which are closely linked to apoptotic cell death, by simple chemicals. Therefore, to exclude the effects related to apoptosis, we at first determined the concentrations of simple chemicals that induced the cell death of MoDC 24 h after chemical treatment. By evaluating DC viability using propidium iodide (PI) and annexin V staining, we determined the toxic concentrations of each chemical (Fig 1), and defined the maximum concentration that did not induce significant cell death as the sublethal concentration.

Next, we examined CD86 expression on MoDC, which is a representative marker of mature DC. Figure 2, which shows summarized data from six different experiments using MoDC from different donors, shows that, at the sublethal concentration, most of the contact sensitizers, $NiCl_2$, $MnCl_2$, thimerosal, and DNCB, but none of the non-sensitizers, $ZnCl_2$, SDS, or BC, induced the augmentation of CD86 on MoDC. Unexpectedly, another contact sensitizer, HCHO, did not induce the augmentation of CD86 expression.

$NiCl_2$ and DNCB lowered the GSH/GSSG ratio in MoDC at different kinetics and at different intensities Next, we examined the GSH/GSSG ratio of human MoDC at various time intervals after stimulation with sublethal concentrations of DNCB and $NiCl_2$ (Fig 3). DNCB reduced the GSH/GSSG ratio immediately after stimulation and the reduction continued for 120 min. On the other hand, $NiCl_2$ increased the GSH/GSSG ratio immediately after stimulation and then gradually decreased it at least by 120 min after the stimulation. In addition, the reduction of the GSH/GSSG ratio was much smaller in MoDC treated with $NiCl_2$ than in MoDC treated with DNCB.

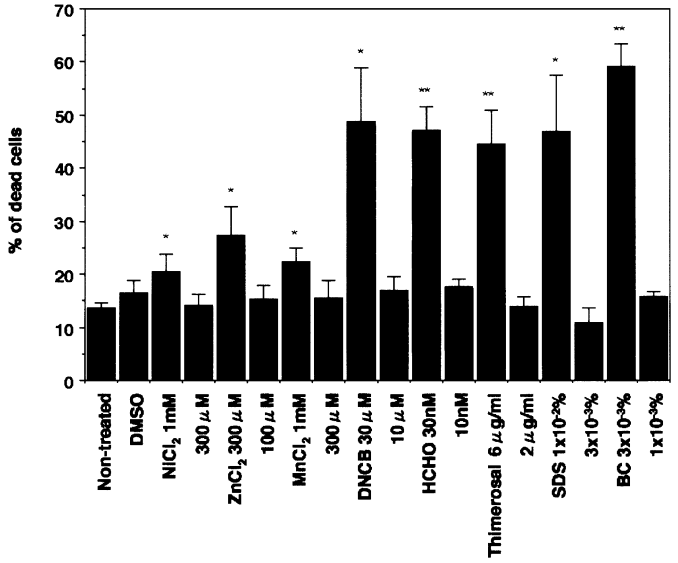

Figure 1
Concentration of simple chemicals to induce cell death of monocyte-derived dendritic cells (MoDC). MoDC were treated with various concentrations of contact sensitizers or non-sensitizers for 24 h. Then the total percentage of both apoptotic cells (annexin V (+)) and necrotic cells (propidium iodide (PI) (+)) was assessed by flow cytometry. The mean ± SEM of the percentage of annexin V-positive cells or PI-positive cells from seven independent experiments is shown. Asterisks indicate significance ($^{**}p<0.01$, $^{*}p<0.05$) for the difference between stimulated cells and non-treated cells.

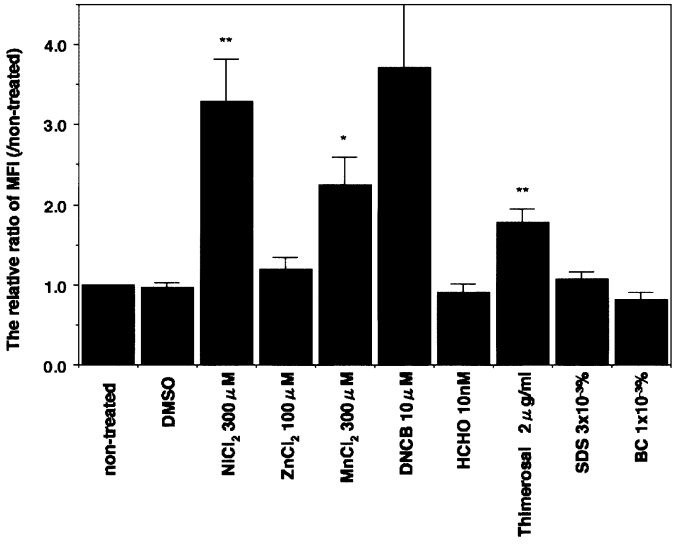

Figure 2
Some contact sensitizers at sublethal concentrations upregulated the expression of CD86 in monocyte-derived dendritic cells (MoDC). MoDC were either unstimulated or stimulated with sublethal concentrations of contact sensitizers, $NiCl_2$, $MnCl_2$, 2,4-dinitrochlorobenzene (DNCB), HCHO, and thimerosal, or non-sensitizers, $ZnCl_2$, SDS, and benzalkonium chloride (BC) for 48 h. The surface expression of CD86 antigen was analyzed by flow cytometry. The mean ± SEM of the relative mean fluorescence intensities (MFI) was determined from four to six independent experiments. Asterisks indicate significance ($^{**}p<0.01$, $^{*}p<0.05$) for the difference between stimulated cells and relevant control cells, non-treated, or those treated with dimethyl sulfoxide (DMSO).

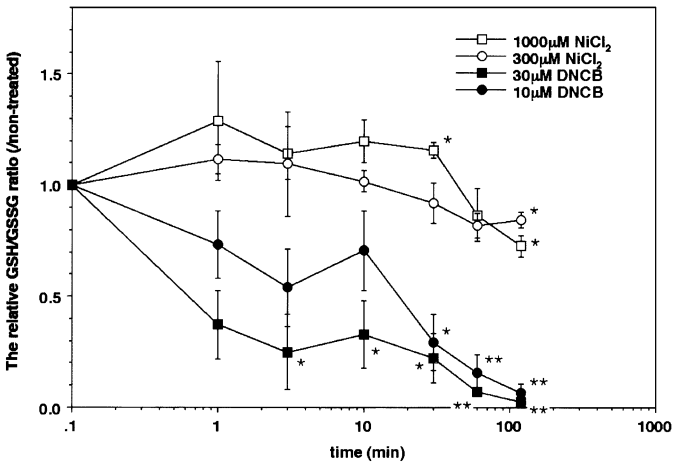

Figure 3
2,4-dinitrochlorobenzene (DNCB) and $NiCl_2$ reduced the reduced/oxidized glutathione (GSH/GSSG) ratio in monocyte-derived dendritic cells (MoDC) at different kinetics. MoDC were cultured with 300 μM or 1 mM of $NiCl_2$ or 10 or 30 μM of DNCB for 0, 1, 3, 10, 30, 60, and 120 min. After stimulation with the chemicals, the GSH/GSSG ratio in MoDC was assessed using colorimetric assays by the GSH reductase-DTNB recycling procedure. The relative GSH/GSSG ratio was calculated by the following formula. The relative GSH/GSSG = the GSH/GSSG ratio in MoDC treated with chemicals/that in non-treated MoDC. The mean ± SEM of the relative GSH/GSSG ratio from three independent experiments is shown. Asterisks indicate significance ($^{**}p<0.01$, $^{*}p<0.05$) for the difference between stimulated cells and the non-treated control.

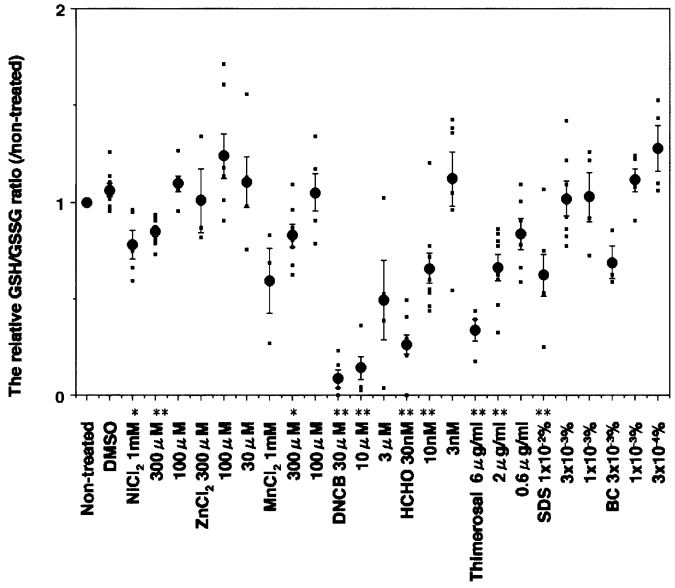

Figure 4
All contact sensitizers, but not non-sensitizers, at sublethal concentrations decreased the reduced/oxidized glutathione (GSH/GSSG) ratio in monocyte-derived dendritic cells (MoDC). MoDC were either unstimulated or stimulated with three concentrations of contact sensitizers, $NiCl_2$, $MnCl_2$, 2,4-dinitrochlorobenzene (DNCB), HCHO, and thimerosal, or non-sensitizers, $ZnCl_2$, SDS, and benzalkonium chloride (BC) for 2 h. The three concentrations of each chemical consisted of a lethal concentration, sublethal concentration, and 1/3 of the sublethal concentration. Intracellular GSH and GSSG were measured using colorimetric assays by the GSH reductase-DTNB recycling procedure. The relative GSH/GSSG ratio was calculated as described in Materials and Methods. Each dot corresponds to the ratio obtained from different experiments and the mean ± SEM of the relative GSH/GSSG ratio is also presented. Asterisks indicate significance ($^{**}p<0.01$, $^{*}p<0.05$) for the difference between stimulated cells and the non-treated control.

All contact sensitizers, but not non-sensitizers, at sublethal concentrations lowered the GSH/GSSG in MoDC Since both DNCB and $NiCl_2$ significantly reduced the GSH/GSSG ratio 120 min after stimulation, we examined the GSH/GSSG ratio 120 min after stimulation with three different concentrations of sensitizers, $NiCl_2$, $MnCl_2$, DNCB, thimerosal, and HCHO, and non-sensitizers, $ZnCl_2$, SDS, and BC (Fig 4). We selected three different concentrations including a lethal concentration, sublethal concentration, and 1/3 of the sublethal concentration. All contact sensitizers reduced the GSH/GSSG ratio at the sublethal concentration. The reduction of the GSH/GSSG ratio was dependent on sensitizers and was always smaller in MoDC treated by metals such as $NiCl_2$ or $MnCl_2$ than in MoDC treated by DNCB, HCHO, or thimerosal. On the other hand, none of the contact sensitizers or non-sensitizers reduced the GSH/GSSG at 1/3 of the sublethal concentration, whereas all contact sensitizers and some non-sensitizers, i.e., SDS, reduced the GSH/GSSG ratio at the lethal concentration.

Contact sensitizers phosphorylated p38 MAPK of MoDC at different kinetics Next we examined the phosphorylation of p38 MAPK in MoDC after sensitizer or irritant stimulation. Since $NiCl_2$ and DNCB reduced the GSH/GSSG ratio at different kinetics, we examined the phosphorylation of p38 MAPK at various time intervals after stimulation. Coincident with the kinetics of the change in the GSH/GSSG ratio, at both the toxic and sublethal concentrations DNCB induced strong phosphorylation of p38 MAPK immediately after stimulation, and then gradually decreased pp38 MAPK, whereas $NiCl_2$ gradually induced the phosphorylation of p38 MAPK by 60 min after stimulation (Fig 5*a* and *b*). $MnCl_2$ also induced pp38 MAPK at kinetics similar to that of $NiCl_2$ (data not shown). The other sensitizers, HCHO and thimerosal, also induced pp38 MAPK with kinetics similar to that of $NiCl_2$. In contrast, BC induced pp38 MAPK only slightly and much less than all sensitizers at both the toxic and sublethal concentrations, although the induction was statistically significant. On the other hand, SDS at the sublethal concentration induced pp38 MAPK far less than the sensitizers, whereas it strongly phosphorylated p38 MAPK at the toxic concentration (Fig 5*a* and *c*).

NAC increased the GSH/GSSG ratio and suppressed the phosphorylation of p38 MAPK and the augmentation of CD86 expression by the sensitizers Next, we added NAC in the culture of MoDC with or without stimulation by the simple chemicals. NAC increased the GSH/GSSH ratio of non-stimulated MoDC. In addition, it significantly recovered the reduction of the GSH/GSSG ratio of MoDC stimulated with sensitizers (Fig 6). Interestingly, NAC suppressed the phosphorylation of p38 MAPK induced by DNCB or $NiCl_2$, although they reduced the GSH/GSSG ratio with different kinetics (Fig 7*a* and *b*). Furthermore, NAC suppressed the augmentation of CD86 induced by either DNCB or $NiCl_2$ (Fig 8).

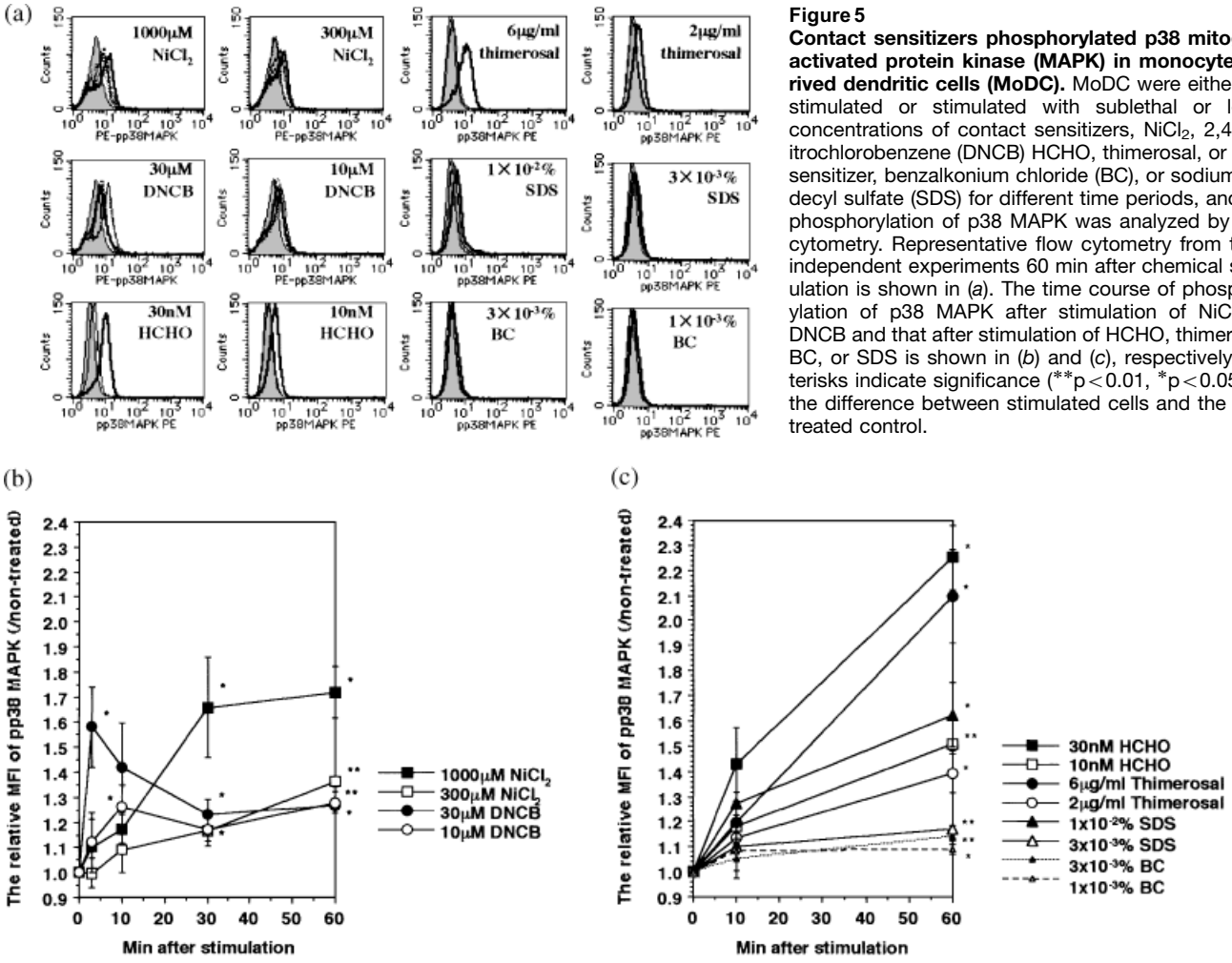

Figure 5
Contact sensitizers phosphorylated p38 mitogen-activated protein kinase (MAPK) in monocyte-derived dendritic cells (MoDC). MoDC were either unstimulated or stimulated with sublethal or lethal concentrations of contact sensitizers, $NiCl_2$, 2,4-dinitrochlorobenzene (DNCB) HCHO, thimerosal, or non-sensitizer, benzalkonium chloride (BC), or sodium dodecyl sulfate (SDS) for different time periods, and the phosphorylation of p38 MAPK was analyzed by flow cytometry. Representative flow cytometry from three independent experiments 60 min after chemical stimulation is shown in (*a*). The time course of phosphorylation of p38 MAPK after stimulation of $NiCl_2$ or DNCB and that after stimulation of HCHO, thimerosal, BC, or SDS is shown in (*b*) and (*c*), respectively. Asterisks indicate significance ($^{**}p<0.01$, $^{*}p<0.05$) for the difference between stimulated cells and the non-treated control.

All contact sensitizers, but not the non-sensitizers, reduced the GSH/GSSG ratio in human monocytic cell-line THP-1 cells at the sublethal concentration Finally, we examined whether the change of the GSH/GSSG ratio and the phosphorylation of p38 MAPK induced by sensitizers constitute a biological response specific to DC or can be observed in monocytes. Accordingly, we examined the GSH/GSSG ratio in human monocytic cell-line THP-1 cells 2 h after stimulation with three concentrations of the four contact sensitizers $NiCl_2$, DNCB, thimerosal, and HCHO, and the non-sensitizers SDS and BC. Again, even using THP-1 cells instead of MoDC, we could obtain similar results. Namely, all sensitizers except for HCHO significantly reduced the GSH/GSSG ratio (Fig 9).

Discussion

In this study, we hypothesized that sensitizers induce redox or oxidative stress in DC, which subsequently stimulates p38 MAPK. Therefore, we at first examined the GSH/GSSG ratio in MoDC stimulated with simple chemicals, because glutathione is the most abundant intracellular thiol-based antioxidant. The results clearly demonstrated that all the sensitizers reduced the GSH/GSSG ratio in MoDC and phosphorylated p38 MAPK in a concentration-dependent manner. The most remarkable changes in these parameters were detected at the toxic concentration. On the other hand, Kuhn *et al* (1998) and Aiba *et al* (1997) have demonstrated that DC maturation induced by chemicals, such as the augmentation of CD86 expression or that of pro-inflammatory cytokine production, is most remarkable at a sublethal concentration. In the stimulation by some haptens, DC transduce the redox imbalance into their activation and their apoptosis via the phosphorylation of p38 MAPK. Recently, we have reported similar observations in DC irradiated with ultraviolet B (UVB). Namely, relatively lower doses of UVB irradiation, 50–100 J per m^2, activated DC, whereas doses more than 100 J per m^2 induced their apoptosis. Furthermore, both the activation and apoptosis of DC were significantly suppressed by the inhibitor of p38 MAPK. Furthermore, the treatment with NAC, which suppressed the reduction of the GSH/GSSG ratio in MoDC, suppressed both the phosphorylation of p38 MAPK and the augmentation of CD86 expression by MoDC. These data support the role of the redox imbalance as one of the triggering factors for DC maturation by at least some sensitizers.

In this study, there were significant differences in the reduction of the GSH/GSSG ratio and phosphorylation of p38 MAPK between SDS and BC. BC even at the toxic concentration did not reduce the GSH/GSSG ratio significantly, or phosphorylated p38 MAPK far less than the sensitizers.

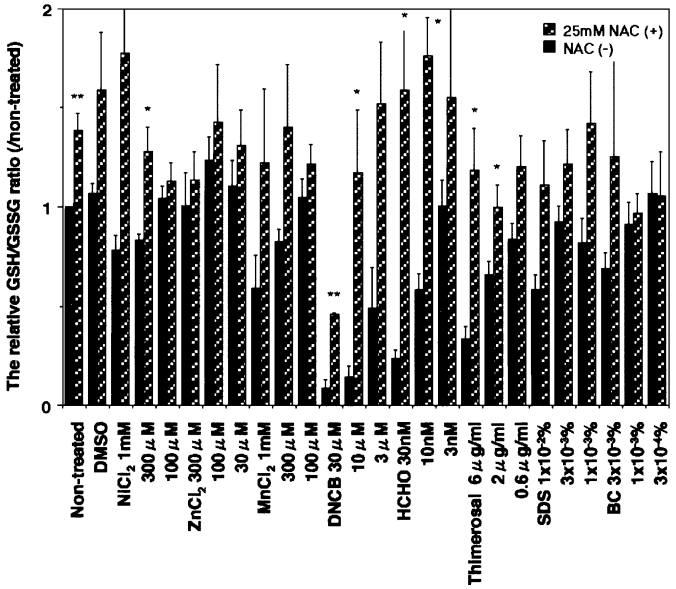

Figure 6

***N*-acetyl-L-cysteine (NAC) increased the reduced/oxidized glutathione (GSH/GSSG) ratio in monocyte-derived dendritic cells (MoDC) with or without chemical stimulation.** To examine the effects of the antioxidant NAC, we added 25 mM NAC to the culture of MoDC 30 min prior to stimulation by the chemicals. Two hours after culture, the relative GSH/GSSG ratio of MoDC was calculated as described in Materials and Methods. The mean ± SEM of the relative GSH/GSSG ratio from three to seven independent experiments is shown. Asterisks indicate significance ($^{**}p<0.01$, $^{*}p<0.05$) for the difference between MoDC treated with NAC and those untreated with NAC.

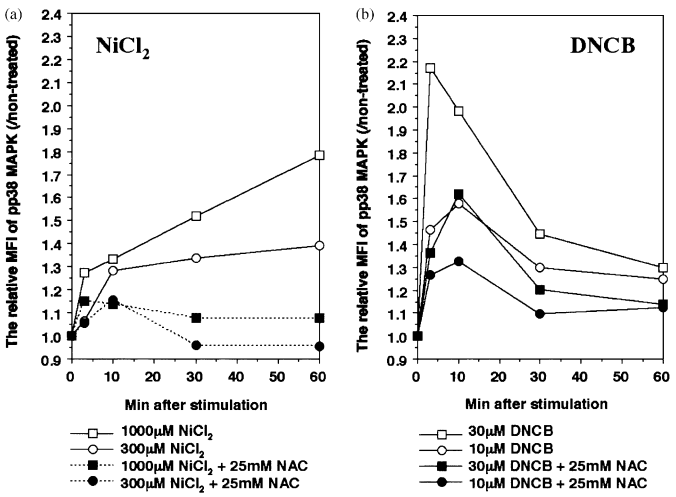

Figure 7

***N*-acetyl-L-cysteine (NAC) suppressed the phosphorylation of p38 mitogen-activated protein kinase (MAPK) in monocyte-derived dendritic cells (MoDC) stimulated with the contact sensitizers.** MoDC were pretreated with 25 mM NAC for 30 min and then cultured with 300 μM or 1 mM of $NiCl_2$ (*a*) or 10 or 30 μM of 2,4-dinitrochlorobenzene (DNCB) (*b*). After 0–60 min of culture, the phosphorylation of p38 MAPK was analyzed by flow cytometry. The relative GSH/GSSG ratio was calculated as described in Materials and Methods. This is a representative flow cytometry of two independent experiments.

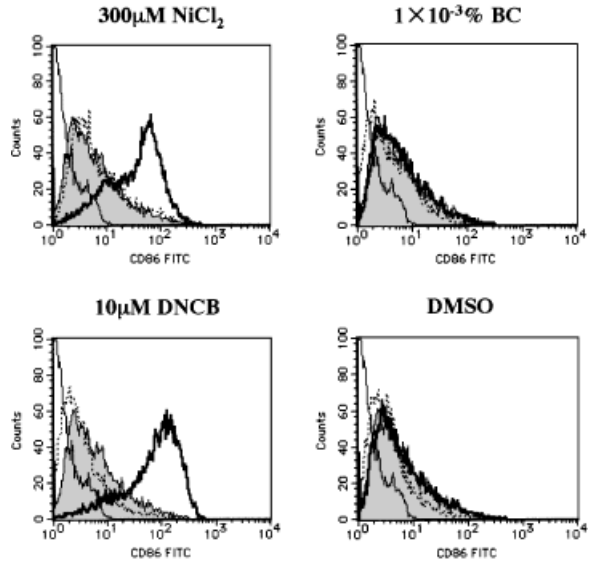

Figure 8

***N*-acetyl-L-cysteine (NAC) suppressed the augmentation of CD86 expression in monocyte-derived dendritic cells (MoDC) stimulated with the contact sensitizers.** MoDC were pretreated with 25 mM NAC for 30 min and then cultured with or without 300 μM of $NiCl_2$, 10 μM of 2,4-dinitrochlorobenzene (DNCB), 1×10^{-3}% benzalkonium chloride (BC), or 0.01% dimethyl sulfoxide (DMSO). After 2 d of culture, their surface expression of CD86 was analyzed by flow cytometry. The histogram of chemical-treated MoDC in the absence or the presence of 25 mM NAC is expressed as a thick solid line or a solid line, whereas a shaded line or a dotted line indicates the histogram of non-treated dendritic cell (DC) or that of DC stained with an isotype control antibody, respectively. This is a representative flow cytometry of three independent experiments.

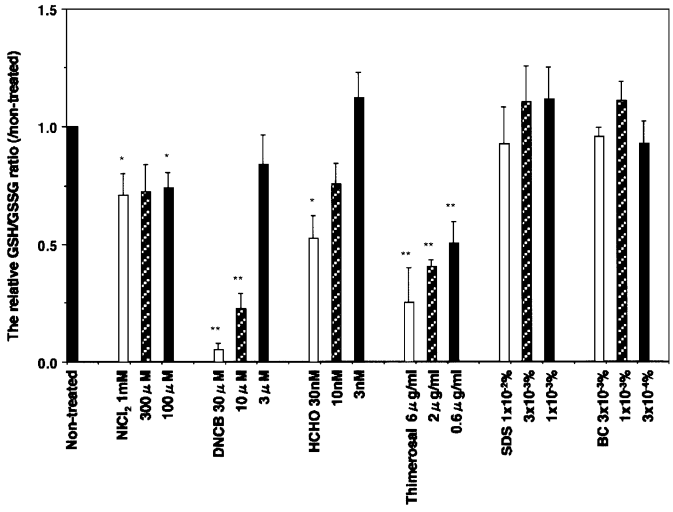

Figure 9

Most contact sensitizers, but not non-sensitizers, at sublethal concentrations lowered the reduced/oxidized glutathione (GSH/GSSG) ratio in human monocytic cell-line THP-1 cells, and the effect that was abrogated by *N*-acetyl-L-cysteine (NAC). Human monocytic cell-line THP-1 cells were either unstimulated or stimulated with three concentrations of contact sensitizers, $NiCl_2$, 2,4-dinitrochlorobenzene (DNCB), HCHO, and thimerosal, or non-sensitizers, SDS and benzalkonium chloride (BC) for 2 h, and then, the GSH/GSSG ratio was measured by the same procedure as shown in Fig 4. At sublethal concentrations, all contact sensitizers except for HCHO, but none of the non-sensitizers, lowered the GSH/GSSG ratio in THP-1 cells. The means ± SEM of the GSH/GSSG ratio was determined in five independent experiments. Asterisks indicate significance ($^{**}p<0.01$, $^{*}p<0.05$) for the difference between stimulated cells and the non-treated control.

In contrast, SDS at the toxic concentration reduced the GSH/GSSG ratio significantly and phosphorylated p38 MAPK more than several sensitizers at the sublethal concentration. Recently, it has been demonstrated that irritant chemicals induced the release of ATP from keratinocytes (Mizumoto *et al*, 2002, 2003) and that ATP can stimulate DC via their purinergic receptors (Schnurr *et al*, 2000; la Sala *et al*, 2001, 2002; Panther *et al*, 2001). According to reports by Mizumoto *et al* (2002, 2003), there was a significant difference in ATP release between SDS and BC. Namely, 0.01% of SDS released approximately 2.8 μM ATP from cultured keratinocytes, whereas 3×10^{-3} M of BC released only 80 nM ATP. These data suggest that the lethal concentration of SDS but not that of BC stimulated MoDC via ATP that was released from the MoDC themselves, although it must be examined whether the data obtained from keratinocytes can be extrapolated to DC. Furthermore, it was also reported that ATP generates ROS (Sauer *et al*, 2001) and stimulates p38 MAPK (Loomis *et al*, 2003).

Recently, Bruchhausen *et al* (2003) have demonstrated that NAC abrogates tyrosine phosphorylation in monocytes stimulated with several sensitizers, and also blocks the phosphorylation of p38 MAPK by MoDC stimulated with TNCB by preventing the binding of TNCB to proteins. In addition, based on their data showing that radical scavengers could not prevent tyrosine phosphorylation in MoDC stimulated with sensitizers, they suggested that ROS appear to be unimportant for the induction of DC activation by sensitizers, and that instead the binding of sensitizers to thiol groups induces intracellular signaling events.

In general, the GSSG/2GSH couple provides a very large pool of reducing equivalents. It is considered to be a cellular redox buffer (Schafer and Buettner, 2001). Recently, Pias and Aw (2002) clearly demonstrated that a cellular redox imbalance could be induced by ROS or by thiol oxidants such as diamide without generating ROS. Using a human B-lymphocyte cell line, Werz *et al* (2001) showed that diamide could induce the phosphorylation of p38 MAPK. On the other hand, Filomeni *et al* (2003) have reported that treatment with exogenous non-permeable GSSG, which results in a significant decrease of exofacial cell membrane thiol groups and an intracellular decrement of the GSH content, phosphorylates p38 MAPK. These data suggest that some sensitizers induce a GSH/GSSG imbalance without generating ROS, which is accompanied by the phosphorylation of p38 MAPK and a subsequent augmentation of CD86 expression.

In this study, however, we found that sensitizers induced the reduction of the GSH/GSSG ratio with different kinetics and at different intensities. Namely, DNCB reduced the GSH/GSSG ratio strongly and immediately, whereas $NiCl_2$ decreased it weakly and gradually. Corresponding to these differences in the changes of the GSH/GSSG ratio, the phosphorylation of p38 MAPK induced by $NiCl_2$ was weaker than that by DNCB. These data suggest that the GSH/GSSG imbalance is induced by different mechanisms. It has been reported that DNCB is conjugated with GSH by various glutathione S-transferase isoenzymes and depletes GSH through this mechanism (Awasthi *et al*, 1981). On the other hand, Shi *et al* (1993) demonstrated that nickel (II) generates ROS by reacting with cysteine in an aerobic environment. Therefore, $NiCl_2$ may reduce the GSH/GSSG ratio by producing ROS.

In *in vivo* studies, Schmidt and Chung (1992) examined changes in the levels of GSH and GSSG in mouse skin 12 h after epicutaneous application of several sensitizers, e.g., dinitrohalobenzenes and picryl chloride, and related non-sensitizing analogues. They observed that the elevation of GSSG levels and/or depletion of GSH levels correlated well with the contact allergenic potential. Furthermore, Hirai *et al* (1997) described the changes in GSH metabolism in mouse skin and liver and their role in irritant contact dermatitis and allergic contact dermatitis by DNCB. When mice were challenged with DNCB, cutaneous and hepatic levels of glutathione and free SH groups rapidly decreased in both sensitized mice and unsensitized mice. These results also suggested the crucial role of the reduced GSH/GSSG ratio in the induction of allergic contact dermatitis.

In this study, we could not demonstrate the augmentation of CD86 by MoDC stimulated with one of the representative sensitizers, HCHO, whereas HCHO reduced the GSH/GSSG ratio and phosphorylated p38 MAPK. Since HCHO and thimerosal could induce similar responses in terms of the GSH/GSSG ratio and phosphorylation of p38 MAPK, we have no clear answer as to why only HCHO could not augment the CD86 expression. Interestingly, HCHO could not reduce the GSH/GSSG ratio in THP-1, whereas thimerosal significantly reduced it. These data suggest that HCHO and thimerosal reduced the GSH/GSSG ratio by different mechanisms. Therefore, the stimulation by HCHO may lack a signal critical for the augmentation of CD86 expression.

Finally, as many new 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 keratinocyte cultures, LC cultures, MoDC, DC or monocyte cell lines, co-cultures systems, and human skin equivalents have been reported. Among them, detecting the augmentation of CD86 expression on MoDC or THP-1 may be a promising candidate (reviewed by Ryan *et al*, 2001). It has become clear, however, that some strong sensitizers, such as HCHO, do not necessarily augment the CD86 expression on MoDC, as demonstrated in this experiment, or on THP-1 when exposed to $NiCl_2$ (Ashikaga *et al*, 2002; Yoshida *et al*, 2003). In contrast, this study succeeded in showing that stimulation even with HCHO and $NiCl_2$ resulted in a GSH/GSSG imbalance, which suggests that measuring the GSH/GSSG ratio could be a novel method to identify sensitizing chemicals.

Materials and Methods

Media and reagents The medium used in this study was RPMI-1640 including 25 mM Hepes buffer (Sigma Chemical, St Louis, Missouri) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 1% penicilline, streptomycin, and fungizone antibiotic solution (Sigma Chemical), and 10% fetal calf serum (Bioserum, Canterbury, Vic., Australia) (complete medium). The buffer used for the purification of $CD14^+$ monocytes from peripheral blood mononuclear cells (PBMC) was PBS supplemented with 1% bovine serum albumin (less than 1 ng per mg of detectable endotoxin) (Sigma Chemical) and 5 mM EDTA (MACS buffer). $NiCl_2$, $ZnCl_2$, $MnCl_2$, thimerosal, BC, SDS (Sigma Chemical), DNCB, and for-

malin (HCHO) (Wako Pure Chemicals, Osaka, Japan) were used for the stimulation of MoDC. The endotoxin content of the final dilution used was <30 pg per mL, as determined by the Limulus amebocyte lysate assay (Seikagaku, Tokyo, Japan). We used the following monoclonal antibodies (mAb) for immunostaining: PE-conjugated-anti-CD86 Ab, PE-conjugated isotype-matched mouse control Abs (IgG1 and IgG2a) (PharMingen, San Diego, California), and PE-conjugated anti-phospho-p38 MAPK (T180/Y182) Ab (Becton-Dickinson, San Jose, California). MACS colloidal supermagnetic microbeads conjugated with anti-human CD14 mAb (CD14 microbeads) were purchased from Miltenyi Biotec (Sunnyvale, California). Recombinant human (rh) GM-CSF was a gift from Kirin Brewery (Tokyo, Japan) and rh IL-4 was purchased from Biosource (Camarillo, California). $NiCl_2$, $ZnCl_2$, $MnCl_2$, thimerosal, HCHO, BC, or SDS was solubilized in distilled water, whereas DNCB was solubilized in dimethyl sulfoxide (DMSO) at a concentration of 1 M. The final concentration of DMSO was always less than 0.1% and cultures of MoDC with 0.1% DMSO were also examined as a control. The antioxidant NAC was purchased from Calbiochem (La Jolla, California) and solubilized in distilled water, and then the pH was adjusted to 7.4. This study was approved by 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 MoDC from peripheral blood monocytes and chemical treatment with sensitizers in the presence or absence of NAC PBMC were isolated from heparinized fresh leukocyte-enriched buffy coats from different donors using Lymphoprep (Nycomed Pharma As, Oslo, Norway). After several washes with PBS, 1×10^8 PBMC were treated with 150 μL of CD14 microbeads in 600 μL of MACS buffer at 4°C for 30 min. 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 per mL of rh GM-CSF and 100 ng per mL rh IL-4 for 6 d. One half of the culture medium was changed on days 3 and 6. On the sixth or seventh day, the cells were treated with different concentrations of $NiCl_2$, $ZnCl_2$, $MnCl_2$, thimerosal, HCHO, DNCB, SDS, or BC. To analyze for intracellular glutathione, these cells were cultured for periods from 1 min to 2 h. To examine the effects of the antioxidant NAC, MoDC were exposed to 25 mM NAC 30 min prior to stimulation by the chemicals.

Dead cell detection A total of 1×10^6 cells were incubated with 5 μL of annexin V-FITC in binding buffer (Clonetech Laboratories, Palo Alto, California) for 15 min, and then washed and resuspended in binding buffer before the addition of 0.5 mg per mL PI solution. Apoptotic cells (annexin V-positive and PI-negative cells) and necrotic cells (PI-positive cells) were then quantified by flow cytometry.

Immunophenotyping of DC Forty-eight hours after treatment with the chemicals, the surface expression of CD86 antigen was analyzed by flow cytometry. Cell staining was performed using a PE-conjugated anti-CD86 (10 μg per mL) or PE-conjugated isotype control (10 μg per mL) Ab. After washing with PBS supplemented with 1% BSA and 0.02% NaN_3 (FACS buffer), the cells were analyzed by FACScan using CellQuest software (Becton-Dickinson). Dead cells were gated out after staining with 0.5 mg per mL PI solution.

Quantification of intracellular GSH and GSSG The 1×10^6 MoDC or THP-1 cells were collected by pipetting and washed twice with cold PBS. The cell pellet was immediately lysed with 200 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 for 5 min to lyse the cells. Thereafter, 10 μL of 0.1 N HCl and 10 μL of 5% sulfosalicylic acid (Wako Pure Chemicals) were added. After centrifugation at 12,000 $\times$ *g* for 5 min, the supernatants were collected for GSH and GSSG assay. The total cellular GSH concentration was assayed using a Total Glutathione Quantification Kit (Dojindo Molecular Technologies, Gaithersburg, Maryland), according to the manufacturer's instructions, which were based on the GSH reductase-DTNB recycling procedure previously reported (Tietze, 1969; Buchmuller-Rouiller *et al*, 1995). The GSSG concentration was assayed according to the method by Sacchetta *et al* (1986). Briefly, 2 μL of 2-vinylpyridine (Wako Pure Chemicals) was added to 100 μL of the cell lysate supernatant, mixed at room temperature for 1 min, and the pH was adjusted to 7.5. Subsequently, the reaction mixture was allowed to stand at room temperature for 60 min to form a measuring sample for GSSG. 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 the total GSH content.

Flow cytometric analysis of phospho-P38 MAPK Ten or 30 min after treatment with the chemicals, the intracellular expression of phospho-p38 MAPK (pp38 MAPK) was analyzed by flow cytometry. We permeabilized cells using Cytofix/Cytoperm solution (BD PharMingen) according to the manufacturer's protocol, and cell staining was performed using a PE-conjugated anti-pp38 MAPK (T180/Y182) (20 μL per 1×10^6 cells) or PE-conjugated isotype control Ab. After washing with PBS supplemented with 1% BSA and 0.02% NaN_3 (FACS buffer), the cells were analyzed by FACScan using CellQuest software.

Statistical analysis The statistical significance of differences in the percentage of dead cells, the relative MFI of CD86 expression, the relative GSH/GSSG ratio, and the relative MFI of phosphorylated p38 MAPK between non-treated MoDC and MoDC treated with chemicals were analyzed using paired Student's *t* test. The statistical significance of differences in the relative GSH/GSSG ratio between non-treated or chemical-treated MoDC or THP-1 and those pretreated with NAC was also analyzed using the paired Student's *t* test.

This study was supported in part by the Japanese Society of Alternatives to Animal Experiments (JSAAE), by Shiseido, and by the 21st COE program of Tohoku University.

DOI: 10.1111/j.0022-202X.2005.23624.x

Manuscript received June 23, 2004; revised October 11, 2004; accepted for publication October 28, 2004

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