
Original Article

The relationship between CD86 and CD54 protein expression and cytotoxicity following stimulation with contact allergen in THP-1 cells

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ABSTRACT — Contact allergens induce the augmentation of cell surface molecules on and release of cytokines from Langerhans cells (LC) in skin sensitization. THP-1 and U937 cell lines, surrogates of LC, were used as analytical tools of this phenomenon recently. In THP-1 cells, contact allergens are reported to induce the phenotypic alteration including the production of pro-inflammatory cytokines and augmentation of cell surface molecules especially at sub-toxic doses. However, the relationship between phenotypic alteration and cytotoxicity is not clear yet. The purpose of this study is to understand the relationship between the protein expression and cytotoxicity induced by contact allergens. First, we observed that the cytotoxicity induced by contact allergens is caused by both apoptosis and necrosis. Apoptosis was preferentially confirmed in stimulation with contact allergens, but non-allergen sodium lauryl sulfate (SLS) hardly induced apoptosis. Moreover, there was no effect to augmentation of protein expression when apoptosis induction pathways were inhibited. Based on these findings, we proposed that the protein expression and cytotoxicity were controlled independently. Next, oxidative stress was found to be generated by contact allergens at the early phase, and this regulated the protein expression and cytotoxicity at least partially. Finally, the humoral factors from dead cells induced by dinitrochlorobenzene (DNCB) were exposed to fresh THP-1 cells to confirm whether protein expression depended on cytotoxicity. The protein expression was not induced. Altogether, these results suggest that cytotoxicity induced by contact allergens may result in apoptosis and may also be stimulated in parallel with protein expression through an intracellular signal or signals.

Key words: THP-1, Skin sensitization, CD86, CD54, Cytotoxicity, Apoptosis

INTRODUCTION

Allergic contact dermatitis, or skin sensitization, is an inflammatory reaction controlled by the immune response. Skin sensitization is composed of two important phases, induction phase and elicitation phase. In the induction phase, there are several key steps: Percutaneous absorption of the hapten, binding of hapten to proteins in the skin to become the allergen, and allergen recognition by the Langerhans cell (LC). Then, the LC becomes the activated form, moves to the draining lymph node, and presents the antigen to antigen-specific T lymphocytes. To investigate whether a certain chemical has a potential to induce skin sensitization, rodent models, for example guinea pigs or mice, have been used (OECD, 1992, 2002). However, recent changes in regulatory requirements (e.g.,

EU testing and marketing bans) and more stringent animal welfare guidelines have lead to the development of alternative methods to evaluate skin sensitization. Therefore, many researchers are actively pursuing the mechanisms of skin sensitization and trying to develop alternative methods based on these mechanisms.

One of the important steps in the induction phase of skin sensitization is the activation of LC. Through the LC activation, expression of cell surface molecules (e.g. CD86, CD54, CD40, and CD83) and release of cytokine (TNF- α , IL-8, IL-1 β and IL-6) are induced *in vivo* (Enk and Katz, 1992; Cumberbatch *et al.*, 1996; Ozawa *et al.*, 1996; Verrier *et al.*, 1999). Such phenotypic alteration has been shown in monocyte-derived dendritic cells (Mo-DC) derived from peripheral blood and in the dendritic cells derived from CD34⁺ hematopoietic progeni-

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tor (CD34-DC) (Aiba *et al.*, 1997, 2003; Coutant *et al.*, 1999; De Smedt *et al.*, 2001, 2002; Staquet *et al.*, 2004). In addition, THP-1 (Human acute monocytic leukemia cell line), U937 (Human leukemic monocyte lymphoma cell line), and MUTZ-3 (Human acute myelomonocytic leukemia cell line) are actively used to clarify the mechanism for skin sensitization because these cells give similar responses to contact allergen as LC or dendritic cells (DC). We have previously reported that the expression of the surface molecules such as CD86, CD54, and CD40, and the production of cytokines such as TNF- α and IL-8 were induced after THP-1 cells are exposed to representative contact allergens 2,4-dinitrochlorobenzene (DNCB) and Nickel sulfate (NiSO₄) (Sakaguchi *et al.*, 2006, 2009; Miyazawa *et al.*, 2007; Nukada *et al.*, 2008). Also, it has been reported that expression of CD86 and the production of IL-1 β and IL-8 are induced in U937 cells when exposed to sensitizers, such as 2,4,6-Trinitrobenzene sulfonic acid (TNBS), DNCB, Eugenol (EU), Citral and so on (Python *et al.*, 2007). In addition, cell signaling events inducing such phenotypic alterations were investigated using THP-1 cell line (Miyazawa *et al.*, 2008a, 2008b). These phenotypic alterations are expected to be the indicators for the identification of contact sensitizers. And, both cell lines THP-1 cells and U937 cells have been widely used as analytical tools in determining the mechanism of LC activation in skin sensitization because of the easy maintenance and broad accessibility (dos Santos *et al.*, 2009).

In the examination of DC and surrogate cell lines, the activation of the cells, such as the augmentation of CD86 expression, is reported to be strongly induced by doses of contact allergens causing 10-40% cytotoxicity (Kühn, *et al.*, 1998; Aiba, *et al.*, 1997). Sakaguchi *et al.* (2009) analyzed the expression correlation of CD86/CD54 and cell viability when induced by several contact allergens in THP-1 cells. In the report, the authors evaluated 21 contact allergens and 8 non allergens, and showed that CD86 and CD54 expression was strongly induced at the doses that caused 10% to 40% cytotoxicity. The authors report a relationship between the augmentation of CD86 and CD54 expression and cytotoxicity induced by contact allergens.

In general, cytotoxicity is cell death and occurs by either apoptotic or necrotic mechanisms. Apoptosis, also known as programmed cell death, forms a shrinkage of the nucleus and the organelles, fragmentation of DNA, shrinkage of the whole cell and the formation of apoptotic body through a specific signaling. Necrosis, also known as mechanical cell death, occurs because of external stimuli with expansion of the cell and the organelles, resulting

in a lysis of the cell (Majno and Jolis, 1995). Previously, it was shown that electrophiles induce necrosis and apoptosis. However, the cytotoxicity induced by the contact allergen in THP-1 cells is not well understood. Moreover, it is uncertain what the influence of cytotoxicity is to protein expression and the cytokine production. The aim of this study is to clarify the response of the THP-1 cell in greater detail and its relationship between protein expression and cytotoxicity.

MATERIALS AND METHODS

Cell culture

THP-1 cells from American Type Culture Collection (Manassas, VA, USA) were cultured in RPMI1640 containing 25 mM HEPES buffer and L-glutamine (Invitrogen Corp., Carlsbad, CA, USA), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen Corp.), 0.05 mM 2-mercaptoethanol and 1% penicillin-streptomycin (Invitrogen Corp.).

Reagents

Table 1 shows the test chemicals used in this study and their abbreviations. Physiological saline (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) and dimethyl-sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA) was used as the solvent for the chemical exposure. FITC-conjugated Annexin V (Annexin V-FITC, BD PharMingen, San Diego, CA, USA), propidium iodide (PI, Sigma-Aldrich), and 7-Amino-Actinomycin D (7-AAD) (BD PharMingen) were used for the analysis of apoptosis and necrosis. The FITC-conjugated anti-CD86 antibodies (Isotype: IgG1 κ) (BD PharMingen), anti-CD54 antibodies (Isotype: IgG1 κ) (DAKO, Glostrup, Denmark), Isotype control antibodies (Mouse IgG1) (DAKO), and Phycoerythrin (PE)-conjugated anti-CD86 antibodies (Isotype: IgG1 κ) (BD PharMingen) were used for the protein expression analysis. Z-VAD-fmk (Promega, Madison, WI, USA), a pan-caspase inhibitor, was used to determine extent of apoptosis inhibition. Antioxidant substance n-acetyl-L-cysteine (NAC, Wako Pure Medicine Industries, Ltd., Osaka, Japan) was used for the reduction of 2' 7' - Dichlorofluorescein diacetate (DCFH-DA, Sigma-aldrich) in the analysis of intracellular reactive oxygen species (ROS) formation.

THP-1 cell chemical exposure

Test chemicals were dissolved in the solvents shown in Table 1. Final concentration of DMSO did not exceed 0.2% and did not have an influence on the outcome. THP-1 cells were cultured at 1×10^6 cells per sample at 37°C,

Table 1. List of the chemicals used to determine the relationship between CD86 and CD54 protein expression and cytotoxicity following contact allergen stimulation

	Chemicals	Abbreviations	CAS#	LLNA	Vehicles	Purchased from
Sensitizer	2,4-dinitrochlorobenzene	DNCB	97-00-7	Positive	DMSO	Sigma-aldrich
	Propyl gallate	PG	121-79-9			
	Phenylacetaldehyde	PAA	122-78-1			
	Eugenol	EU	97-53-0			
Non-sensitizer	Sodium lauryl sulfate	SLS	151-21-3	False positive	Saline	
	Lactic acid	LA	50-21-5	Negative		

for a prescribed duration. The duration of test chemical exposure was 24 hr in the analysis of CD86/CD54 expression, and 6, 12 or 24 hr in the analysis of apoptosis/necrosis. The concentrations of the test chemical were selected between non-toxic to sub-toxic doses, with a moderate decrease in cell viability. For the analysis of cell viability, THP-1 cells were exposed to various concentrations of chemicals for 24 hr, collected, washed with 0.1% bovine serum albumin containing phosphate buffer saline (FACS buffer), and PI was added. Cell viability was measured by flow cytometry (FACSCalibur, Becton Dickinson, San Jose, CA, USA).

In the inhibition assay, THP-1 cells were pre-treated with Z-VAD-fmk dissolved in DMSO at final concentrations of 2, 5, or 10 μ M (for inhibition of apoptosis) for 1 hr and then cells were exposed to test chemicals including Z-VAD-fmk. For determining role of antioxidant, THP-1 cells were pre-treated with NAC dissolved in HEPES buffer at a concentration of 5 mM (for antioxidant) for 1 hr, and then exposed to the test chemicals including NAC.

The measurement of the amount of intracellular ROS was conducted with THP-1 cells treated with 10 μ M DCFH-DA for 30 min at 37°C in the dark. Cells were washed with fresh medium and then cultured with fresh medium containing the test chemicals. After 1 hr, cells were collected, washed by FACS buffer and analyzed using flow cytometry.

Transwell assay

THP-1 cells were treated with 5 or 10 μ g/ml DNCB for 3 hr as described above, washed with fresh medium, and re-suspended in fresh medium. The cells were then cultured in Transwell (1 μ m pore, BD Falcon; upper well) at 1×10^6 cells/well and deemed as the “donor cells”. Additionally, fresh THP-1 cells were added to the lower wells of the 24 well plate (lower well) at 1×10^6 cells/well and deemed as the “acceptor cells”. Transwells were set on the 24 well plate containing acceptor cells, the donor cells

and acceptor cells were co-cultured across the transwell for 24 hr, and then analyzed for CD86 and CD54 expression.

Detection of apoptosis and necrosis

The detection of apoptosis and necrosis were performed by double staining with Annexin V-FITC and PI according to the manufacturer directions. Briefly, cells were washed by FACS buffer twice and the Annexin V binding buffer was replaced with a 1×10^5 cells/100 μ l. Five μ l of Annexin V-FITC and PI (final concentration; 50 μ g/ml) were added to the cell suspension, and cells were incubated for 15 min at room temperature in the dark. After incubation, 400 μ l Annexin V binding buffer was added to each sample and a total of 10,000 cells were analyzed by FACSCalibur shown as Fig. 1A. Cells were classified into living cells (Annexin V-/PI-), early apoptotic cells, (Annexin V+/PI-), dead cells (PI+), and frequencies for each groupings was calculated. For three-color immunofluorescence assay, Annexin V-FITC, PE-anti-CD86 antibodies, and 7-AAD were used. Briefly, cells were washed by FACS buffer twice and the Annexin V binding buffer was replaced with a 1×10^5 cells/100 μ l. Five μ l of Annexin V-FITC, 6 μ l of PE-CD86 antibody, and 7-AAD (final concentration; 2.5 μ g/ml) were added to the cell suspension, and cells were incubated for 15 min at room temperature in the dark. After incubation, 400 μ l Annexin V binding buffer was added to each sample and a total of 10,000 cells were analyzed by FACSCalibur.

Analysis for the CD86 and CD54 expression

Flow cytometric analysis of CD86 and CD54 expression was performed as described by Sakaguchi *et al.* (2009) with a mean fluorescence intensity (MFI) used for the calculation of the CD86 and CD54 expression level. The corrected MFI, which was calculated from MFI of CD86 or CD54 minus MFI of isotype control, was the expression level for each sample.

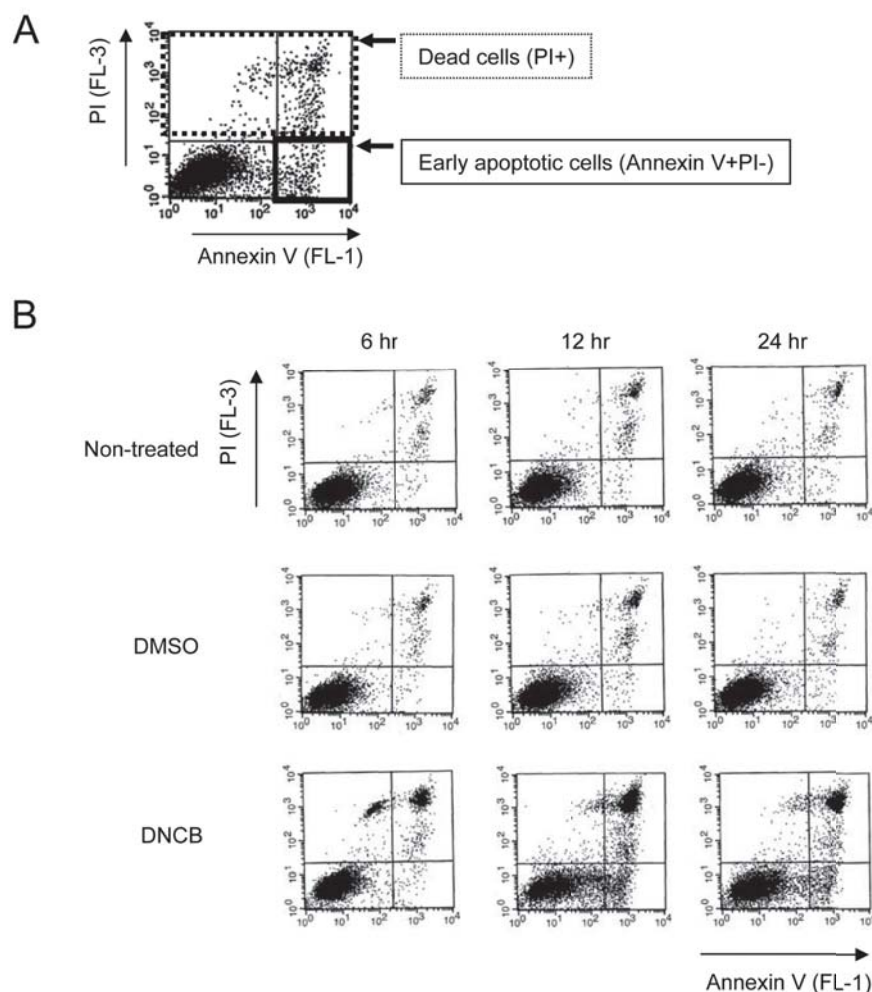


Fig. 1. Change of apoptosis and dead cells after stimulation with DNCB. THP-1 cells were exposed to DNCB at 5 $\mu\text{g/ml}$ for 6, 12, or 24 hr, respectively, and then analyzed using Annexin V-FITC and PI. For control analysis, cells were not exposed to any chemical (control) or were treated with DMSO (vehicle) as the DNCB-treated cells. A) Double staining analysis. B) Dot plot for control, DMSO, or DNCB-treated cells.

Immunoblotting

Total amount of Ask1 and phosphorylated Ask1 were detected by using immunoblotting technique as described by Miyazawa *et al.* (2008a). Cells were lysed in ice-cold lysis buffer (RIPA buffer, Santa Cruz BioTechnology, Santa Cruz, CA, USA), and after centrifugation, the cell extracts were subjected to SDS-PAGE and analyzed by immunoblotting as described by Miyazawa *et al.* (2008a) with minor modification. The membranes were probed with antibodies to phosphoAsk1 (provided by Prof. Ichijo, University of Tokyo) and Ask1 (Santa Cruz). The blots were detected with ECL Plus (GE Healthcare, Waukesha, WI, USA).

Statistics

Each experiment was independently executed at least three times. A Student's t-test was used to ascertain statistical significance and p-values less than or equal to 0.05 or 0.01 were considered statistically significant.

RESULTS

Apoptosis is induced preferentially by the contact allergen stimulation

THP-1 cells were exposed to a variety of contact allergens and non-allergens and the cytotoxicity was analyzed to understand the role of the cytotoxicity in the induction of CD86 and CD54 marker expression by the various

chemicals. We hypothesized that the cytotoxicity induced by the allergenic chemicals would be due to apoptosis or necrosis. Therefore, the study employed double staining using Annexin V-FITC recognizing phosphatidylserine, one of the markers of early apoptosis, and PI probing to indicate dead cells including necrosis. The cytotoxicity profiles of the flow cytometry of the non-treated cells, DMSO-treated cells, and 5 $\mu\text{g}/\text{ml}$ DNCB-treated cells are shown in Fig. 1B and the ratio of early apoptotic cells (Annexin V+/PI-) and dead cells (PI+) for each chemical are shown in Fig. 2. All four contact allergens (DNCB, PG, PAA, and EU) statistically increased the fraction of the early apoptotic cells compared to vehicle control. The maximum increases in early apoptotic cells was $9.0 \pm 1.5\%$ at 24 hr, $15.6 \pm 2.0\%$ at 6 hr, $19.3 \pm 5.1\%$ at 12 hr, and $6.0 \pm 1.4\%$ at 12 hr for DNCB, PG, PAA, and EU, respectively. In contrast, the non-sensitizers (SLS and LA) also induced apoptosis compared to vehicle control, but the magnitude of the induction was less for non-allergens than for sensitizers. In the apoptosis induction, time to maximum fraction of apoptotic cells was different for each test chemical. PG, PAA, and EU induced

maximum increases at 6 hr, 12 hr, and 12 hr, respectively; though DNCB took a full 24 hr. Overall, the fraction of dead cells increased as time passed regardless of the presence of allergen or non-allergen. However, the fraction of early apoptotic cells at each time point was fewer than the fraction of dead cells in the allergen response as well as in the non-allergen response. Although both allergens and non-allergens induced cytotoxicity (dead cells), apoptosis was induced preferentially by allergens as indicated by the greater response in apoptotic cells.

Allergen induction of CD86 and CD54 protein expression does not influence apoptosis

To investigate whether apoptosis, which is one of the cytotoxicity mechanisms in allergen activation, was affected by CD86 and CD54 protein expression, the chemical inhibitor of apoptosis, Z-VAD-fmk, was used. Z-VAD-fmk inhibits several caspases that are key regulators of apoptosis. The fraction of early apoptotic cells from DMSO-treated cells without inhibitor was $0.45 \pm 0.14\%$ and with inhibitor (10 μM) was $0.42 \pm 0.12\%$ (Fig. 3). There was little change with or without

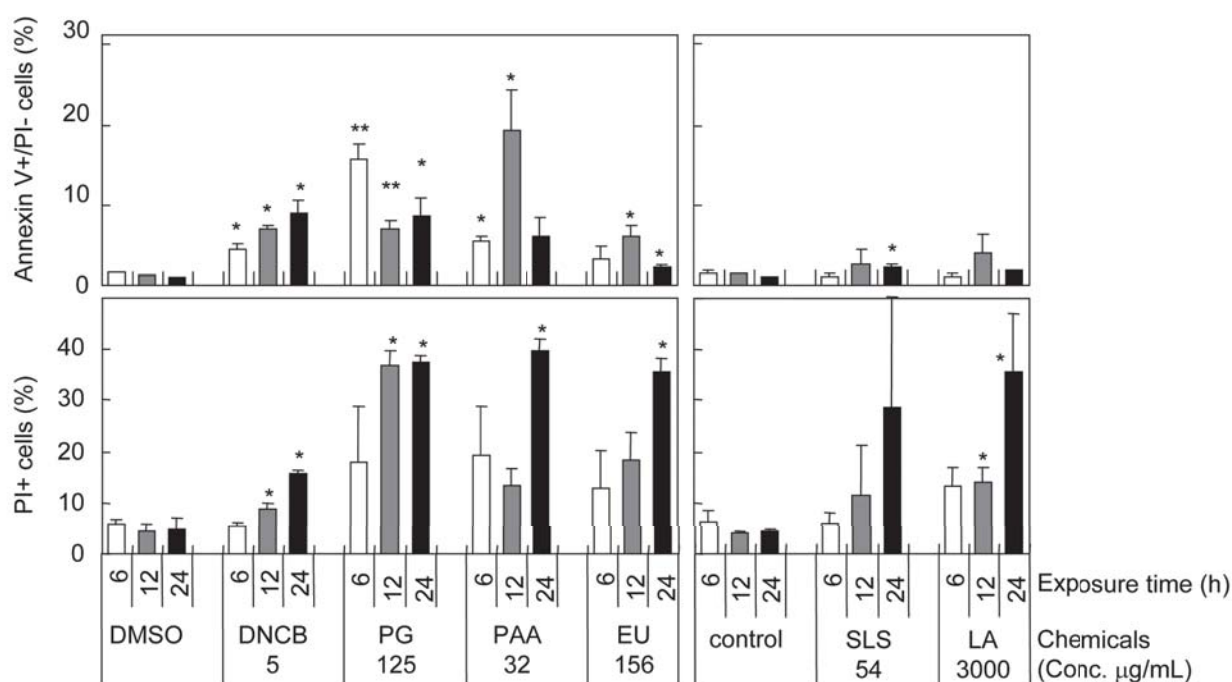


Fig. 2. Characteristics of cell death in cells treated with sensitizer or non-sensitizer. THP-1 cells were exposed to various concentrations of four sensitizers (DNCB, PG, PAA and EU) and two non-sensitizers (SLS and LA). After cultivation, cells were analyzed using double staining with Annexin V-FITC and PI. The upper figure shows the ratio of early apoptotic cells (Annexin V + / PI -) and the lower figure shows the ratio of dead cells including necrotic cells (PI+). Mean $\pm$ S.D. ($n = 3$). Statistical significance was calculated by Student's t-test between the vehicle control and test chemicals (* $P < 0.05$, ** $P < 0.01$).

inhibitor, indicating that 10 μM of the inhibitor itself did not affect THP-1 cells. But in the presence of 5 $\mu\text{g/ml}$ DNCB, the ratio of the early apoptotic cells in treated cells without inhibitor was $6.91 \pm 0.47\%$ and with inhibitor (10 μM) was $3.18 \pm 0.35\%$; confirming that cell viability decreased to less than 90% with DNCB stimulation without an inhibitor. When an inhibitor was used, the cell viability was greater than 90%, therefore the inhibitor prevented cell death (Fig. 3B). Relative expression of CD86 and CD54, shown as corrected MFI, were compared for DNCB-treated cells, where a statistically significant augmentation of CD86 and CD54 expression occurred but protein expression did not change after treatment with Z-VAD-fmk at any concentration (Figs. 3C and 3D). This provides evidence that the induction pathway of apoptosis was not affected by the protein expression, and apoptosis and protein expression might be regulated by independent pathways.

The expression of CD86 and CD54 in early apoptotic cells and normal cells induced with DNCB was also examined. In the 3-color immunofluorescence assay, Annexin V-7-AAD- "normal" cells, seen 24 hr after stimulation with DNCB, strongly expressed CD86. In contrast, Annexin V+7-AAD+ early apoptotic cells did not (Fig. 4). This result concludes that the protein expression is induced in the normal cells and not in apoptotic cells following stimulation with contact allergen DNCB.

The contact allergen induces oxidative stress at the early stage of the exposure

Miyazawa *et al.* (2008a) has reported that the one of the induction pathways of CD86 and CD54 expression includes p38MAPK. However, there is no information about the induction pathway of cytotoxicity induction by contact allergens. Hence, a pathway for cytotoxicity induced by contact allergens and its influence on protein expression were analyzed.

Electrophilic contact allergens are known to cause a stress response at an early stage of the exposure. In this section, an analysis of whether the contact allergen caused oxidative stress, and whether the oxidative stress influenced protein expression or cytotoxicity was conducted in THP-1 cells. Fig. 5A shows the extent of ROS generation in THP-1 cells following exposure to the contact allergens DNCB, PAA, and EU. All allergens induced ROS formation (Fig. 5A). Strong ROS generation was confirmed in the presence of 5 $\mu\text{g/ml}$ DNCB. Furthermore, phosphorylation of Ask1, which is part of the oxidative stress response, was analyzed. Contact allergens induced the phosphorylation of Ask1, though there were differences in the extent of induction among the allergens

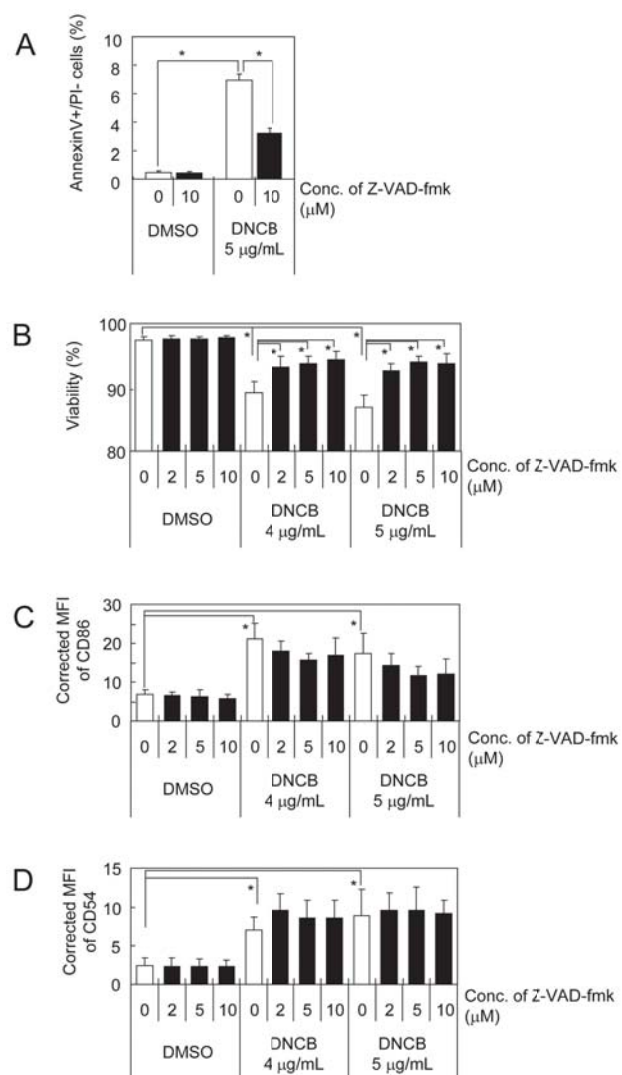


Fig. 3. The effect of apoptosis-inhibition on cytotoxicity and protein expression induction in THP-1 cells exposed to contact allergen. THP-1 cells were pretreated with Z-VAD-fmk at restrictive concentrations (2, 5, 10 μM) for 1 hr. Afterwards, inhibitor-treatment was assessed in cells exposed to DNCB (4, 5 $\mu\text{g/ml}$) for 24 hr. White bar shows the results without inhibitor and black bar shows the results with inhibitor. A) the ratio of Annexin V+PI- (the early apoptotic cells), B) Cell viability, C) Corrected CD86 MFI, D) Corrected CD54 MFI. Mean $\pm$ S.D. (n = 4) Statistical significance was calculated by Student's t-test (*P < 0.05).

(Fig. 5B). For DNCB, both a strong induction of phosphorylation of Ask1 was observed with a strong generation of ROS. For EU, the induction in phosphorylation of Ask1 was as strong as that of DNCB, but ROS formation

Protein expression and cytotoxicity in a contact allergen response

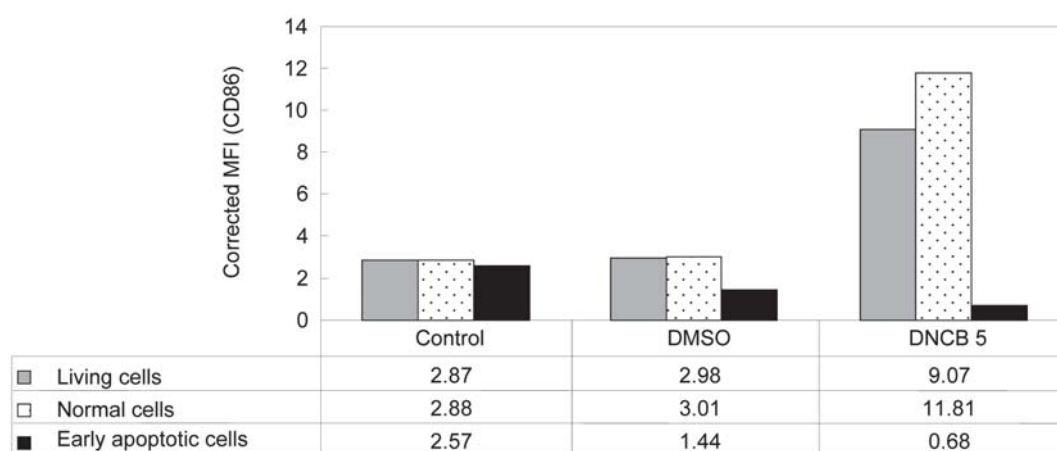


Fig. 4. The induction of protein expression in living cells, normal cells, and early apoptotic cells stimulated with contact allergen DNCB. Cells were incubated with DNCB at 5 $\mu\text{g/ml}$ for 24 hr. Cells were stained with Annexin V-FITC, PE-CD86 antibody, and 7-AAD. Annexin V + or - / 7-AAD - cells were named "living cells", Annexin V - / 7-AAD - cells were named "normal cells", and Annexin V + / 7-AAD - cells were named "early apoptotic cells". The protein expression was described as corrected MFI for CD86.

was lower than DNCB. As a result, the allergen induction mechanism for inducing the oxidative stress response would not be simple but complex, and the mechanism seems to depend on the chemical property,

Association of contact allergen induced oxidative stress with protein expression and cytotoxicity

We next investigated how the induced protein expression and the cytotoxicity in the THP-1 cell changed when the oxidative stress was suppressed by using an antioxidant substance. In this study, NAC was used as the antioxidant. After pretreatment of THP-1 cells with NAC for 1 hr, the extent of protein expression was analyzed (Fig. 5C). In the results, both the expression of CD86 or CD54 proteins and the cytotoxicity suppression observed with DNCB and PAA were statistically significant lower with NAC treatment (Fig. 5C). A statistically significant change of either CD86 or CD54 by NAC treatment was not confirmed with EU though the cytotoxicity tended to be decreased. Therefore, we considered that the oxidative stress induced by contact allergens during the early phase was preferentially associated with the cytotoxicity.

Humeral factors from dead cells induced by contact allergen stimulation did not affect the augmentation of CD86 and CD54 expression in fresh THP-1 cells

Both cytotoxicity and protein expression appear to be regulated by the oxidative stress pathway, at least par-

tially. Therefore, we investigated whether both protein expression and cytotoxicity were responses regulated by direct contact allergen stimulation or whether protein expression was regulated by the cytotoxicity in allergen response. In the transwell assay, the donor cells stimulated with 5 $\mu\text{g/ml}$ DNCB showed an increase of CD86 and CD54 expression and moderate cytotoxicity (about 25%) while 10 $\mu\text{g/ml}$ DNCB induced only a strong cytotoxicity (cell viability less than 50%) (Fig. 6A). The donor cells were expected to generate dead cells during co-culture and that some factors would be released from the dead cells to the culture medium. In the current study, acceptor cells were also analyzed and there were no differences between the co-culture with DMSO-treated cells and the co-culture with DNCB-treated cells on both protein expression and cytotoxicity (Fig. 6B). This result suggests that humeral factors from dead cells do not effect the protein expression of normal THP-1 cells.

DISCUSSION

This study was based on the following hypothesis: Cytotoxicity and protein expression specifically induced by contact allergens are regulated independently. The level of allergen needed to induce cytotoxicity will also induce the augmentation of protein expression. To clarify whether the hypothesis is correct or not, we investigated the relationship between protein expression and cytotoxicity induction in THP-1 cells stimulated with contact allergen.

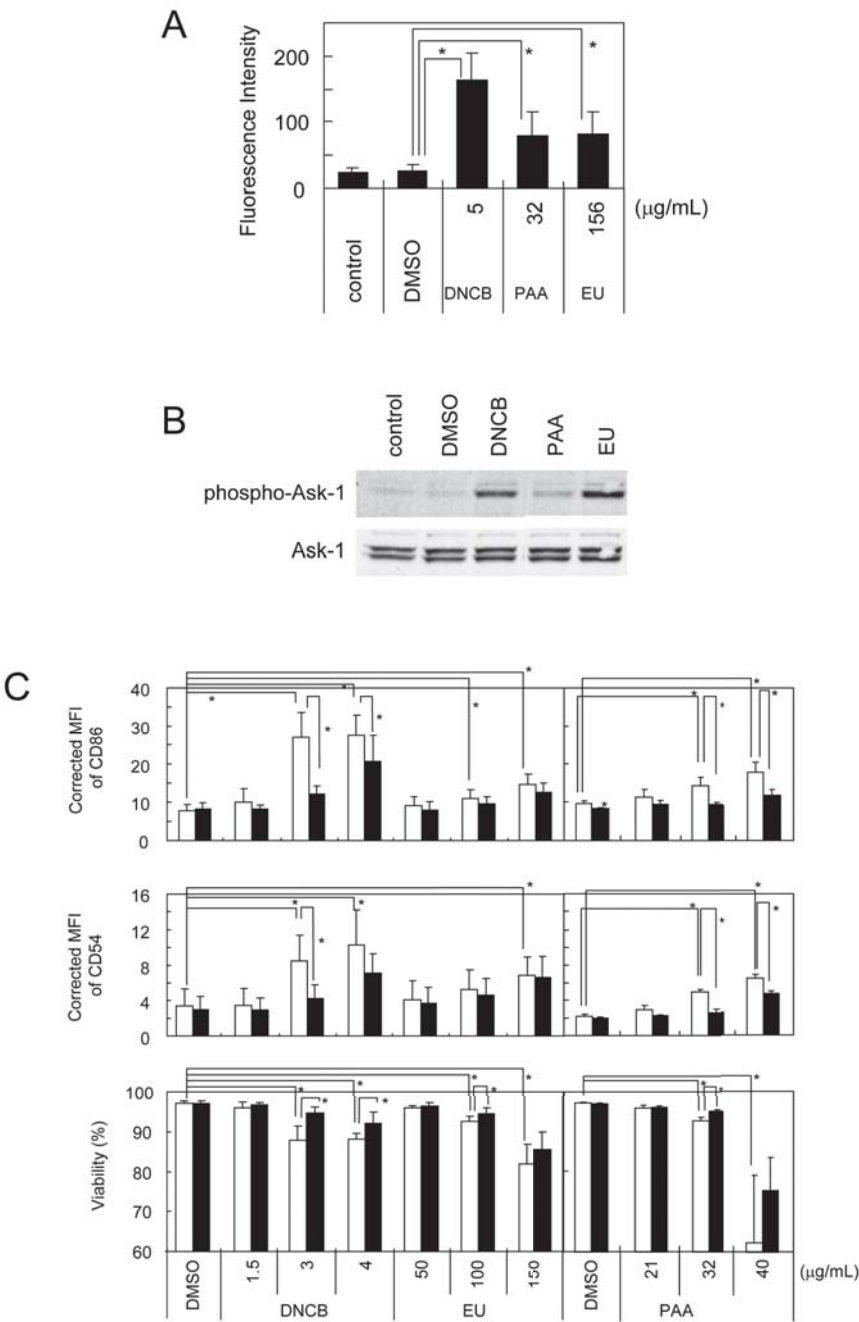


Fig. 5. The induction of oxidative stress in THP-1 cells following stimulation with contact allergens and the effect of NAC pre-incubation on THP-1 cell responses. A) The accumulation of intracellular ROS using DCFH-DA. THP-1 cells were pretreated with DCFH-DA (10 μM), washed, and then exposed to representative contact allergens, DNCB, PAA, and EU for 1 hr. The fluorescence intensity indicates the level of ROS accumulation. Mean $\pm$ S.D. ($n = 4$) B) The phosphorylation of Ask1 after stimulation with DNCB, PAA, and EU. C) THP-1 cells were pre-treated with NAC at 5 mM for 1 hr. Cells were then exposed to DNCB, EU, and PAA at various concentrations. Afterwards, the protein expression and cytotoxicity were analyzed. White bar shows the results without NAC pretreatment and black bar shows the results following NAC pretreatment. Data is reported as the mean $\pm$ S.D. (DNCB, $n = 7$; PAA, $n = 7$; PAA, $n = 4$). Statistical significance was calculated by Student's t-test (* $P < 0.05$).

Protein expression and cytotoxicity in a contact allergen response

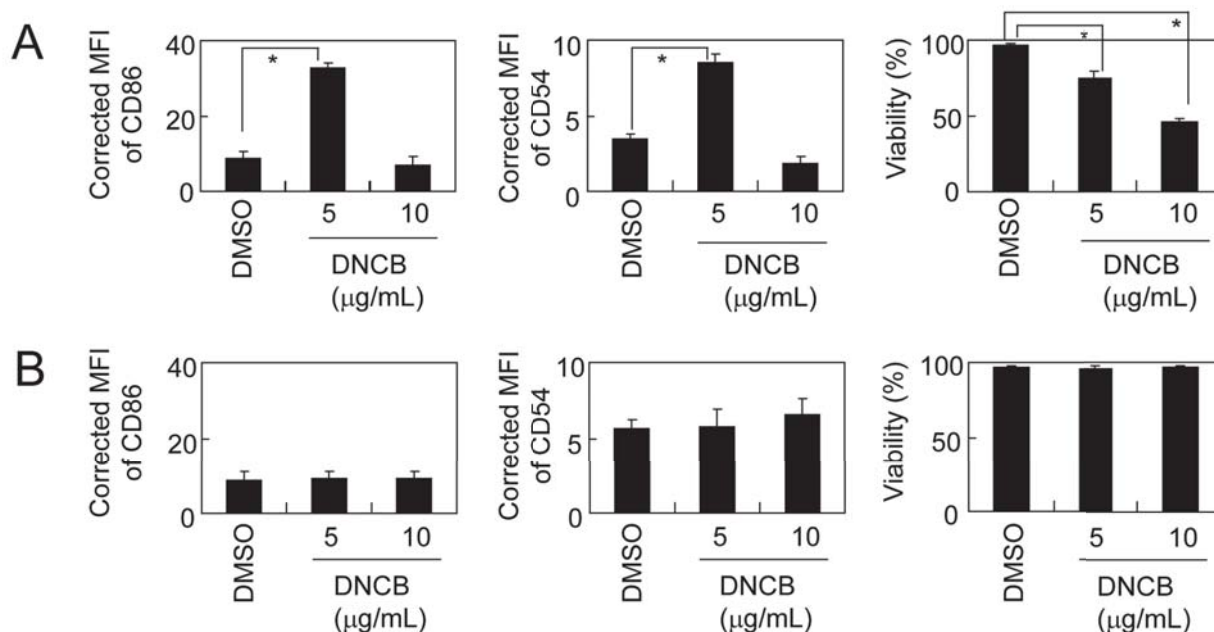


Fig. 6. Donor cell study: The effect of dead cell contents from THP-1 cells treated with contact allergen on the expression of CD86 and CD54 or cytotoxicity in THP-1 cells. THP-1 cells were exposed to DNCB (5 or 10 µg/ml) for 3 hr. Culture medium was changed to fresh medium, and then stimulated-cells (donor cells) were co-cultured with fresh THP-1 cells (acceptor cells) across the transwell for up to 24 hr. After incubation, donor cells and acceptor cells were analyzed for protein expression and cell viability as shown in A or B, respectively. Each data was described as mean $\pm$ S.D. for 3 independent assays. Statistical significance was calculated by Student's t-test (* $P < 0.05$).

Previously, many researchers have stated that the phenotypic alteration in dendritic cells is strongly induced especially at sub-toxic doses (Hulette *et al.*, 2005; Straube *et al.*, 2005; Sakaguchi *et al.*, 2009). Because non-allergens exposure induces cytotoxicity but not dendritic cell phenotypic alterations, the relation between phenotypic alteration and cytotoxicity was suggested to be associated with contact allergen exposure. In the current study, THP-1 cells were selected as our analytical tool and as an alternate to LC to help clarify this relationship. We examined how cytotoxicity was related to the mechanism of CD86 and CD54 induced expression with various contact allergens.

We confirmed that apoptosis was preferred in the contact allergen response of dendritic cells. The apoptosis induction pathways like the downstream caspase pathway did not affect protein expression of CD86 and CD54 because the augmented expression of CD86 and CD54 caused by exposure to allergens was not disrupted by a caspase inhibitor. Therefore, caspase appears not to be crucial to protein expression but involved in apoptosis through cytotoxicity in THP-1 cells.

Previously, contact allergens were reported to induce apoptosis in other cells. For instance, treatment of Mo-DC with DNCB or metallic salts decreased protein expression as evident by excessive cytotoxicity and confirmed that apoptosis was induced by stimulation with DNCB, 2,4,6-trinitrochlorobenzene (TNCB), NiCl_2 , and cobalt chloride (CoCl_2) (Manome *et al.*, 1999). In other cells, apoptosis was confirmed with NiSO_4 and DNCB in the U937 cell where the induced expression of CD86 by DNCB was inhibited by Z-VAD-fmk (Ade *et al.*, 2006). Ade *et al.* (2006) showed that apoptosis obstruction does not influence CD86 expression, which also coincided with the current research. These data suggest that contact allergens induce apoptosis through signal pathways different from the pathways that induce protein expression.

In addition, the ratio of early apoptotic cells at each exposure time was less than the ratio of dead cells for each test chemical. In general, cells are at various stages in the apoptosis process and the pattern we observed from Annexin V-PI- (normal), Annexin V+PI- (early apoptotic), to PI+ (late apoptotic) as well as the phenotypic features of the late apoptotic cells are similar to

necrotic cells. In this examination, the rate of PI⁺ cells for each exposure time was larger than the rate of Annexin V+PI⁻ cells, indicating that allergens induce not only apoptosis but also necrosis. Based on the reported findings above, the mechanism of cytotoxicity caused by the contact allergen appears to differ for each allergen and suggest that cytotoxicity is a complex phenomenon which includes both apoptosis and necrosis.

The expression of CD86 and CD54 in Annexin V+7-AAD-cells induced with DNCB was also examined. In the experiment, 7-AAD was used in place of PI. Annexin V-7-AAD “normal” cells, seen 24 hr after stimulation with DNCB, expressed CD86 and CD54. But Annexin V+PI⁻ early apoptotic cells did not (Fig. 4). This might suggest that the augmentation of protein expression does not happen in the cells induced by the process of apoptosis. Moreover, an early apoptotic cell is not observed when THP-1 cells were exposed to high doses of DNCB, resulting in a necrotic cell. Augmentation of CD86 and CD54 protein expression was not observed in the current study when an early apoptotic cell was not obtained 24-hr after exposure. However, expression of these proteins is observed after 12-24 hr when early apoptotic cells are obtained following cells treated with appropriate doses of allergens. Therefore, the data suggest that there is an amount of test chemical needed to induce apoptosis, which then induces protein expression in response to contact allergens.

The induction mechanism of cytotoxicity in THP-1 cells stimulate with contact allergens was unknown, and therefore the intracellular transduction pathways were investigated. Previously, Huang *et al.* (1994) reported that a metallic salt such as NiCl₂, a representative contact allergen, induced the accumulation of intracellular ROS and oxidative stress in the CHO cell. It is well-known that the induction of apoptosis and cell differentiation are the phenotypic changes induced by oxidative stress (Buttke and Sandstrom, 1994). Therefore, we used oxidative stress to understand the induction mechanisms of cytotoxicity after cell stimulation with contact allergens. Additionally, we investigated whether contact allergens induce the accumulation of ROS in THP-1 cells and if so, how oxidative stress regulates protein expression and cytotoxicity. In the current study, the measurement of ROS accumulation and phosphorylation of Ask1, an important molecule regulating stress signaling, were chosen as indicators of oxidative stress. Both responses were confirmed when THP-1 cells were exposed to three major contact allergens; indicating that under the current study conditions contact allergens induce oxidative stress in THP-1 cells. Inhibition of oxidative stress leads to partial suppression of

protein expression and cytotoxicity though the degree of suppression depends on the test chemical. Therefore, oxidative stress appears to be at least partially related to protein expression and cytotoxicity.

In general, ROS, ultraviolet rays, metals, medicines/chemicals, etc. are known to induce an intracellular peroxidative environment and after that, the stress response occurs with a variety of intracellular signaling cascades through thioredoxin and glutaredoxin (Nordberg and Arner, 2001). Other research has shown that the stress response is important for the establishment of skin sensitization *in vivo* and the response to allergens *in vitro* (Natsch and Emter, 2008; Ade *et al.*, 2009). Ade *et al.* (2009) reported that in CD34-DC cells, contact allergen stimulation induces the accumulation of transcription factor Nrf2, which is activated by the electrophile or ROS, following the expression of stress-reactive HMOX-1 and NQO-1 genes.

In the Ade *et al.* (2009) study, antioxidant NAC suppressed the expression of the HMOX-1 and NQO-1 genes after stimulation with 5 μ M DNCB. This dose of DNCB was the same as in our current study and we would have expected a similar result in our cells. Mizuashi *et al.* (2005) reported that after stimulation with DNCB and NiCl₂ in Mo-DC and THP-1 cells; the ratio of GSH/GSSG was changed, indicating an induction redox imbalance. In Mizuashi *et al.* (2005), the inhibition of oxidative stress using NAC induced the increase of the ratio of GSH/GSSG, suppression of p38MAPK phosphorylation, and CD86 expression. These results suggest that ROS production in the present study and change of the ratio of GSH/GSSG in Mizuashi *et al.* (2005) might be related to each other in THP-1 cell activation induced by a contact allergen.

Several investigators have suggested that a redox imbalance is induced not only with ROS accumulation but also with the thiol oxidant, such as diamine, without ROS production (Pias and Aw, 2002; Filomeni *et al.*, 2003). Therefore, this would mediate ROS accumulation. We observed a decreased inhibition effect of NAC treatment on protein expression and cytotoxicity, which would confirm this theory. This suggests that a stress response caused without the accumulation of ROS exists. On the other hand, the accumulation of ROS and the induction of Ask1 phosphorylation were confirmed to occur also in cells exposed to non-allergens (data not shown). However, the non-allergens did not induce protein expression. Therefore, induction of oxidative stress as result from ROS production or Ask1 phosphorylation would not be specific to contact allergens, but a common phenomenon, and induction mechanisms of augmentation of protein

expression and cytotoxicity would be regulated by some machinery other than oxidative stress.

Because contact allergens could bind to nucleophilic material like thiol or amino acids on proteins, allergens may directly affect the functionality of the protein. An example is thioredoxin reductase (TrxR). TrxR plays an important role in oxidative stress, because TrxR reduces not only the disulfide in oxidized thioredoxin (Trx) but also other protein disulfides through the active center of TrxR (Arner *et al.*, 1999) in an intracellular oxidative state. When the thiol (SH) residue of the active center of TrxR binds with an electrophilic compound, TrxR cannot reduce Trx or other proteins. Accumulation of oxidation of TrxR, of course, could induce oxidative stress and lead to phenotypic alterations. Therefore many electrophilic compounds could become inhibitors of TrxR (Nordberg and Arnér, 2001) and induce oxidative stress. DNCB is a known inhibitor of TrxR (Nordberg *et al.*, 1998) and has been reported to influence the function of the protein by acting directly on TrxR (Arnér *et al.*, 1995). Thus, the mechanism that influenced the function of the cell is suggested to occur by oxidant stress through the ROS accumulation.

We used another approach to confirm that cytotoxicity and protein expression were separately induced by allergens. We investigated whether humoral factors from dead cells that had been exposed to contact allergens directly induced protein expression of THP-1 cells. Humoral factors obtained from dead cells treated with high concentrations of DNCB did not induce any cellular responses in receptor cells including CD86 and CD54 expression or cytotoxicity. These results suggest that augmentation of protein expression due to contact allergen exposure does not depend on humoral factors from dead caused by allergen exposure. Miyazawa *et al.* (2008b) reported that the TNF- α from THP-1 cells activated by contact allergens was related to CD86 and CD54 expression. But in contrast, there is no induction of CD86 and CD54 expression after incubation with humoral factors from dead cells. These phenomena suggest that the allergen driven protein expression in our donor studies did not result in sufficient levels of TNF- α to activate THP-1 cells. Yet, Sakaguchi *et al.* (2009) reported that protein induction of CD86 and CD54 could occur with non-toxic doses of allergens; though protein expression especially became strong at sub-toxic doses of contact allergen in THP-1 cells. These observations support the idea that cytotoxicity is not essential for the induction of protein expression and that two different pathways or mechanisms are involved in parallel that yield the phenotype of contact allergen stimulation. Moreover, these phenomena

are controlled, at least partially, by oxidative stress occurring at early stages of the exposure to the allergen.

In conclusion, we found that the phenotypic responses, protein expression and cytotoxicity, observed in THP-1 cells exposed to contact allergens occur in parallel and independently. We also suggested that a threshold effect may be involved *in vitro* since the allergen exposure needs to cause weak or moderate cytotoxicity in order for the phenotypic alterations including CD86 and CD54 expression to occur. *In vivo*, allergens may also have a threshold affect in sensitization. Moreover, the induction phase of sensitization requires a level of mild irritation. The critical amount of allergen involved in sensitization might be related *in vitro* and *in vivo*.

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