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Human Pathology (2015) 46, 1662–1669

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Original contribution

Androgen receptor, androgen-producing enzymes and their transcription factors in extramammary Paget disease☆

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Received 19 May 2015; revised 6 July 2015; accepted 10 July 2015

Keywords:
Extramammary Paget's disease;
Immunohistochemistry;
Androgen receptor;
Androgen-producing enzymes;
Transcription factor

Summary Extramammary Paget disease (EMPD) has been known to frequently express androgen receptor (AR). Therefore, androgens could play roles in the biological behavior of Paget cells. 5α-Reductase (5α-red) types 1 and 2 and 17β-hydroxysteroid dehydrogenase type 5 (17β-HSD5) are pivotal in situ regulators of androgen production in androgen-responsive tissues including androgen-dependent neoplasms. Therefore, in this study, we immunolocalized AR, androgen-producing enzymes, and their transcription factors to assess the state of in situ androgen production and actions and its correlation of invasiveness in EMPD. We studied 51 cases of EMPD with known clinicopathological status. AR, 5α-red1, 17β-HSD5, and β-catenin immunoreactivity was evaluated by using the modified H-score method while cyclin D1, p53, forkhead box protein P1, and a proliferation marker, Ki-67, were quantified using labeling index. The mean scores of AR, 5α-red1, and 17β-HSD5 in invasive EMPD were all significantly higher than noninvasive EMPD ($P < .0001$). Ki-67 labeling index as well as the cyclin D1 score was also significantly higher in invasive than noninvasive lesions of EMPD. These results demonstrated that androgen receptor and androgen-producing enzymes were both associated with cell cycle regulation and subsequently the invasiveness of EMPD lesions and could also indicate those above as potential markers of invasive potentials in EMPD.

1. Introduction

Androgen receptor (AR) has been known to be expressed in extramammary Paget disease (EMPD) with a greater proportion of samples expressing AR than either estrogen receptors (ERs) or progesterone receptors [1]. Therefore, androgens have been proposed to play some roles in the pathogenesis or development of EMPD. 5α-Reductase types

Abbreviations EMPD, extramammary Paget disease; 17β-HSD5, 17β-hydroxysteroid dehydrogenase type 5; 5α-red1, 5α-reductase type 1; 5α-red2, 5α-reductase type 2; AR, androgen receptor; ER, estrogen receptor; CK19, cytokeratin 19; FOXP1, forkhead box protein P1

☆ Competing interests: The authors declare that they have no conflict of interest.

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http://dx.doi.org/10.1016/j.humpath.2015.07.007
0046-8177/

1 and 2 (5α-red1 and 5α-red2) and 17β-hydroxysteroid dehydrogenase type 5 (17β-HSD5) are considered pivotal enzymatic steps regulating in situ androgen production in both normal and pathologic human androgen-responsive tissues [2]. Recently, Kasashima et al [3] reported higher AR and 5α-red1 expression in invasive than noninvasive lesions of EMPD and highlighted that androgen amplification and signaling could possibly accelerate the invasiveness of these Paget cells. However, to the best of our knowledge, the status of the complementary and alternate androgen-generating enzymes, 5α-red2 and 17β-HSD5, has not been examined in EMPD.

In addition to AR and androgen-producing enzymes, the status of related transcription factors could also be important in androgen-related tumor biology, and the analysis of these transcription factors and steroid receptor could provide pivotal information as to predicting clinical behavior of EMPD. The transcription activity of AR has been reported to be regulated by various coactivators and corepressors, as well as by interactions with other transcription factors [4], but in this particular study, we did analyze the transcription factors with known roles in human cancer biology and/or with possible therapeutic interventions under development including cyclin D1, p53, β-catenin, and forkhead box protein P1 (FOXP1). Cyclin D1 and p53 are well-established cell cycle regulators known to act respectively as oncogenic and tumor suppressing factors [5,6]. Cyclin D1 was also reported a potent corepressor of AR in prostate cancer [7]. FOXP1 and β-catenin have both been implicated in cell signaling pathways, with FOXP1 as a tumor suppressor and β-catenin as both an important structural factor and a mediator of Wnt signaling [8,9]. These overlaps with AR have been previously reported in prostate cancer [4,10]. FOXP1 has been recently studied due to its roles as a candidate tumor suppressor gene and as an androgen-regulated transcription factor possibly involved in the tumorigenesis of prostate cancer [4], but its details are not known in EMPD. We also examined the potential roles of cyclin D1, p53, FOXP1, and β-catenin in association with androgen pathways as possible therapeutic targets in EMPD.

Compared to androgen pathways and FOXP1, p53, cyclin D1, and β-catenin have not been studied in EMPD. Therefore, in this study, we evaluated the interactions of androgen pathways and transcription factors using immunohistochemistry of these factors in EMPD cases. We also compared the immunoreactivity of these factors between noninvasive and invasive EMPD cases to determine whether any of these factors could be correlated with invasive potential of EMPD.

2. Materials and methods

2.1. EMPD cases

Our research protocols were approved by the ethics committees at Tohoku University Graduate School of Medicine (Sendai, Japan). Fifty-one specimens (mean age, 74.73 ± 8.94 years; range, 54-95 years) of EMPD including 34 men (mean age, 74 years; range, 54-89 years) and 17 women (mean age, 76 years; range, 55-95 years) were retrieved from surgical pathology files of Tohoku University Hospital (Sendai, Japan). No significant differences in age were noted between genders. Histologically, the tumor cells were characterized by large, atypical neoplastic cells (Paget cells) harboring large nuclei with prominent nucleoli and abundant pale cytoplasm (Fig. 1). Invasive EMPD was histologically defined as Paget cells infiltrated in the dermis [11].

2.2. Immunohistochemistry

The antibodies and positive controls used in our present study are summarized in Table 1. Immunohistochemistry was carried out as previously described [12]. Cytokeratin 19 (CK19) immunoreactivity was used as an immunohistochemical marker of tumor cells of EMPD in this study (Fig. 1) [13].

2.3. Evaluation of immunoreactivity

AR, 5α-red1, 17β-HSD5, and β-catenin immunoreactivity was evaluated according to the modified H-score method as previously reported [12]. The relative immunointensity was scored as follows: 0, negative; 1, weak; 2, intermediate; or 3,

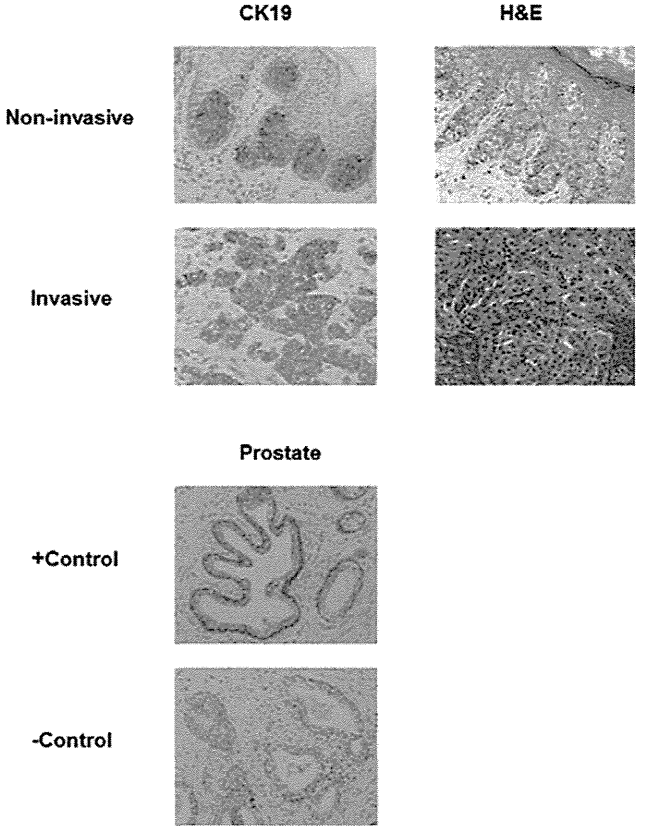

Fig. 1 Hematoxylin and eosin staining and immunohistochemistry of CK19 in noninvasive and invasive lesions of EMPD. Original magnification ×200. +control, positive control; −control, negative control.

Table 1 Summary of primary antibodies used in this study

Antigens	Source	Dilution	Manufacturer	Antigen retrieval	Positive control
AR	Mouse	1:50	Dako	Autoclave	Prostate
17β-HSD5	Mouse	1:200	Sigma	Autoclave	Adrenal gland
5α-red1	Goat	1:1000	Abcam	–	Liver
5α-red2	Rabbit	1:1000	Immunostar	–	Liver
Ki-67	Mouse	1:100	Dako	Autoclave	Tonsil
β-Catenin	Mouse	1:200	Dako	Microwave	Breast cancer
Cyclin D1	Rabbit	1:1	Nichirei	Autoclave	Breast cancer
p53	Mouse	1:200	Novocastra	Microwave	Colon cancer
FOXP1	Rabbit	1:100	Spring Bioscience	Autoclave	Tonsil
CK19	Mouse	1:50	Novocastra	Autoclave	Prostate

strong. The H-score was subsequently generated by adding the percentages of strong staining (3×), the percentages of intermediate staining (2×), and the percentages of weakly staining (1×), giving a possible range of 0 to 300. The immunopositive cells in all the noninvasive and invasive areas for cyclin D1, p53, and Ki-67 were all counted at magnification ×400, in 3 different representative fields randomly. A minimum of 200 cells were counted in each section, and the percentage of immunopositive cells was then calculated (labeling index percent [LI%]). Relative immunointensity was graded as negative (nuclear staining of <10% cells) and positive (staining >10% cells) [14].

2.4. Statistical analysis

The correlations among 5α-red1, 17β-HSD5, cyclin D1, and AR were evaluated using regression analysis. The Student *t* test was used to assess the differences between invasive and noninvasive diseases, and in all the cases examined, $P < .05$ was considered statistically significant.

3. Results

3.1. The status of AR and androgen-producing enzymes in EMPD

Fig. 2A illustrated the example of relatively weak immunoreactivity of AR, androgen-producing enzymes, 5α-red1, and 17β-HSD5 in noninvasive and marked immunoreactivity in invasive EMPD. 5α-red2 immunoreactivity was not detected in any of the cases examined. In Fig. 2B, the mean positive cells of AR, 5α-red1, and 17β-HSD5 was significantly higher in invasive than noninvasive cases ($P < .0001$). There was a significant correlation between AR and 5α-red1 immunoreactivity as well as between AR and 17β-HSD5 immunoreactivity in all cases of EMPD examined ($P < .0001$) (Fig. 3A and B).

17β-HSD5 immunoreactivity was detected in all the cases examined (noninvasive and invasive [100%]), as summarized in Table 2. AR nuclear immunoreactivity was detected in 42 (84%) of the 50 noninvasive cases and in all invasive cases (100%). 5α-red1 immunoreactivity was detected in 49 (98%) of the 50 noninvasive cases and 38 (97%) of the 39 invasive cases.

3.2. Colocalization of AR with other factors examined in EMPD

3.2.1. Cyclin D1

Of interest, the status of cyclin D1 immunoreactivity was significant correlated with that of AR in all the cases of EMPD examined in this study ($P < .0001$) (Fig. 3C). Nuclear cyclin D1 immunoreactivity (Fig. 4A) was detected in all of the noninvasive and invasive specimens (100%) (Table 2) but with a significantly increase in prevalence in invasive ($P < .0001$) compared to noninvasive cases of EMPD (Fig. 4B).

3.2.2. p53

Tumor cells were positive for p53 in 14% of noninvasive cases (7/50), but only 8% (3/39) of invasive cases demonstrated p53 immunoreactivity (Table 2; Fig. 4).

3.3. The status of AR coactivator in EMPD

3.3.1. β-Catenin

β-Catenin immunoreactivity was localized to the tumor cell cytoplasm (Fig. 4A), and positive immunoreactivity was detected in all of the noninvasive and invasive cases examined (100%) (Table 2) with no significant difference between invasive and noninvasive lesions (Fig. 4B).

3.4. The status of androgen-responsive transcription factors in EMPD

3.4.1. FOXP1

FOXP1 was immunolocalized to the nuclei and cytoplasm in noninvasive and invasive cases (Fig. 5A). Ninety-four percent of noninvasive (47/50) and 90% of invasive cases (35/39) were positive for FOXP1 (Table 2). However, there

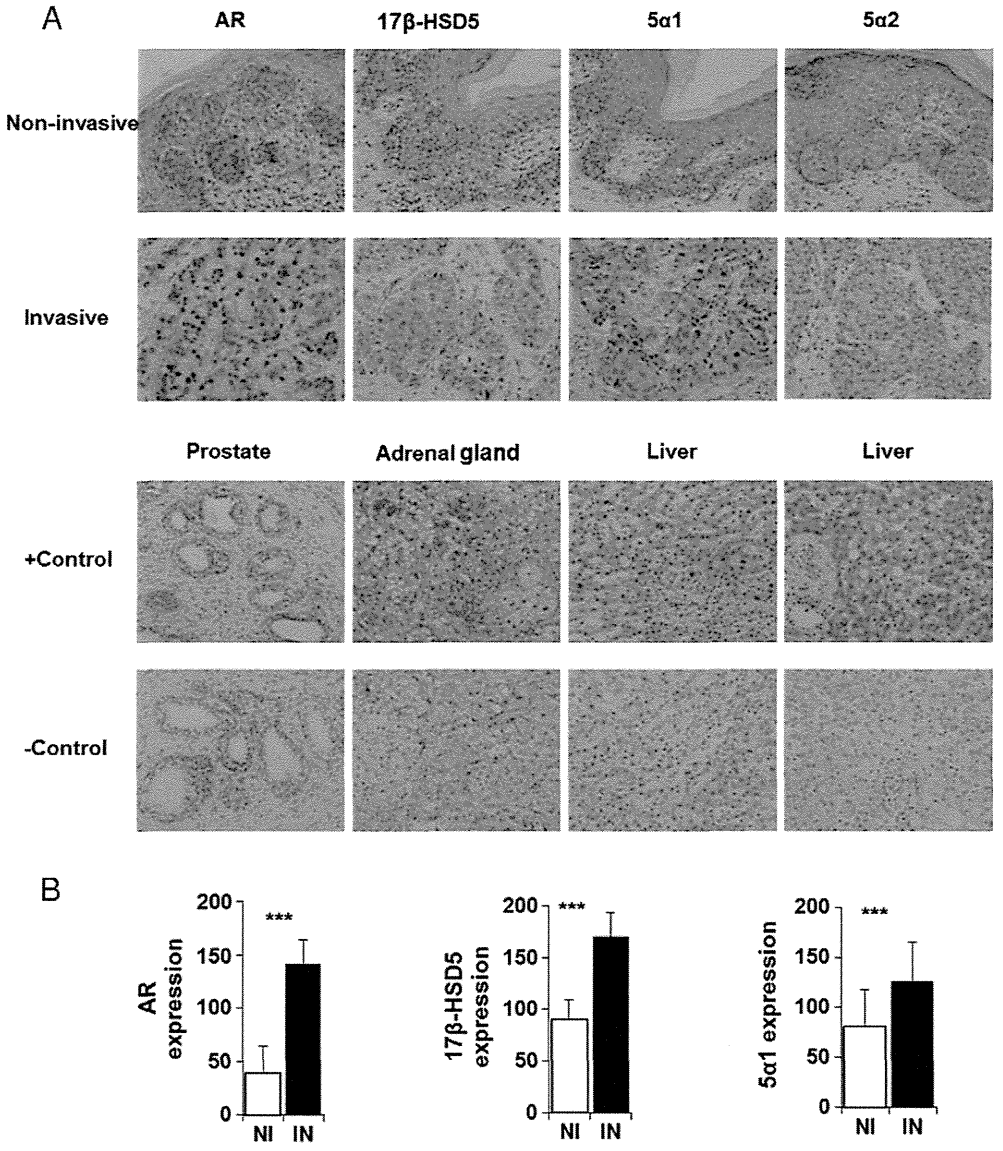

Fig. 2 A, Relatively weak immunoreactivity of AR, 17β-HSD5, and 5α-red1 in noninvasive EMPD and marked immunoreactivity in invasive EMPD. 5α-red2 was immunohistochemically negative in all the cases examined in this study. Original magnification ×200. B, Invasive lesion tended to be positive for AR, 17β-HSD5, and 5α-red2 significantly higher than noninvasive lesion by H-score. +control, positive control; −control, negative control. A significant differences was defined by ***$P < .0001$.

were no significant differences between invasive and noninvasive lesions (Fig. 5B).

3.5. The status of Ki-67 LI in EMPD

Ki-67 LI ranged from 0% to 75% (Table 2; Fig. 5A). Ki-67 LI was significantly higher ($P < .05$) in invasive than in noninvasive cases of EMPD (Fig. 5B).

4. Discussion

Progesterone receptor and ER were reported to be absent but AR to be frequently expressed in EMPD [1]. The presence of AR and 5α-red1 was also reported in EMPD [3], but to the best of our knowledge, this study is the first to demonstrate the presence of the androgen synthesizing pathways (17β-HSD5 and 5α-red2 and 5α-red1) in a large portion of EMPD and the predominance of 5α-red1 over the 5α-red2 subtype. Results of our present study also demonstrated that the status of 17β-HSD5 and 5α-red1 was significantly correlated with AR, suggesting that this is an active pathway of intratumoral androgen synthesis in EMPD. EMP-K1 tumor in the nude mouse from skin metastasis of EMPD was reported to be stimulated by an injection of testosterone, dihydrotestosterone, diethylstilbestrol, and 17β-estradiol, which suggested that this particular transplantable tumor line could be hormone dependent, which could be the basis of possible endocrine treatment of EMPD

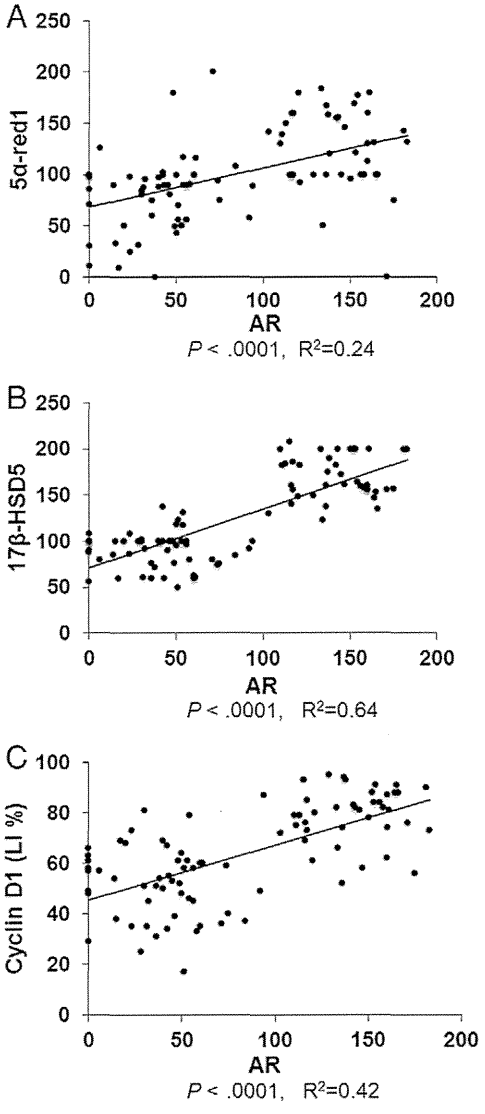

Fig. 3 Correlation analysis of 5α-red1 (A), 17β-HSD5 (B), and cyclin D1 (C) between AR expression in EMPD. A significant differences was defined by ***$P < .0001$.

Table 2 Summary expression of antibodies in non-invasive and invasive of EMPD

	Non-invasive cases		Invasive cases	
	Positive	Negative	Positive	Negative
AR	42 (84%)	8 (16%)	39 (100%)	0 (0%)
5α-red1	49 (98%)	1 (2%)	38 (97%)	1 (3%)
17β-HSD5	50 (100%)	0 (0%)	39 (100%)	0 (0%)
Ki-67	49 (98%)	1 (2%)	38 (97%)	1 (3%)
Cyclin D1	50 (100%)	0 (0%)	39 (100%)	0 (0%)
β-catenin	50 (100%)	0 (0%)	39 (100%)	0 (0%)
FOXP1	47 (94%)	3 (6%)	35 (90%)	4 (10%)
p53	7 (14%)	43 (86%)	3 (8%)	36 (92%)

[15]. In addition, androgen deprivation therapy was also reported to successfully suppress the metastatic lesions of EMPD [16]. Another novel point of our present study is the significantly higher status of AR, 5α-red1, and 17β-HSD5 in invasive EMPD compared with that in noninvasive disease. These findings also did indicate that local androgen production was related to the invasion of Paget cells in EMPD and antiandrogen therapy could be a potential therapeutic regimen for this disease.

EMPD is considered a heterogeneous skin cancer arising from cells of different origin, which results in a degree of confusion in developing a clinical algorithm for the treatment of patients [17]. Therefore, the precise pathogenesis of EMPD has remained virtually unknown. PIK3CA and AKT1 mutations have been recently reported to be more likely to be associated with an invasive phenotype of EMPD, which suggested that an activated PIK3CA/AKT1 pathway could promote the development of this disorder [18]. In addition, in this study, we also examined the status of cyclin D1, p53, β-catenin, FOXP1, and Ki-67 to elucidate the coordinated regulation of multiple transcription factors and regulators in EMPD. Among these factors, cyclin D1 was significantly increased in invasive compared to noninvasive cases of EMPD. An overexpression of cyclin D1 is usually associated with poor prognosis and aggressive and metastatic tumors [19], and cyclin D1 levels are significantly correlated with proliferation [20]. Therefore, its overexpression in aggressive variants of EMPD did suggest its roles as regulating the biological behavior in similar fashion. In addition, Lanzino et al [21] reported that cyclin D1 served as a specific androgen target gene in breast cancer, which could indicate the inhibitory roles of androgens in the process of breast carcinoma cell proliferation. However, it also awaits further investigations to clarify the more detailed correlation between cyclin D1 induction and AR expression in EMPD, especially in its invasive phenotype.

β-Catenin has been reported to play important roles in cell adhesion and signal transduction pathways in many human malignancies [22]. The activation of β-catenin to an oncogenic state is well known to be achieved by inactivation of tumor suppressor adenomatous polyposis coli (APC), direct mutation in the β-catenin gene, or activation of Wnt receptors. Once activated, β-catenin promotes tumor progression through an interaction with its downstream targets [23]. β-Catenin was reported to be present in both mammarian and extramammarian Paget disease. Although it is true that there were no significant differences of the status of β-catenin between invasive and noninvasive diseases in our present study, diffuse cytoplasmic β-catenin immunoreactivity was detected in all the cases of noninvasive and invasive neoplasms. These could also indicate that β-catenin remains as a possible component of cell signaling pathways in this disease. Previous studies have reported an aberrant activation of other components of the β-catenin signaling pathways, such as the Wnt signaling pathway in some cases of Paget disease of the vulva [24]. Functional

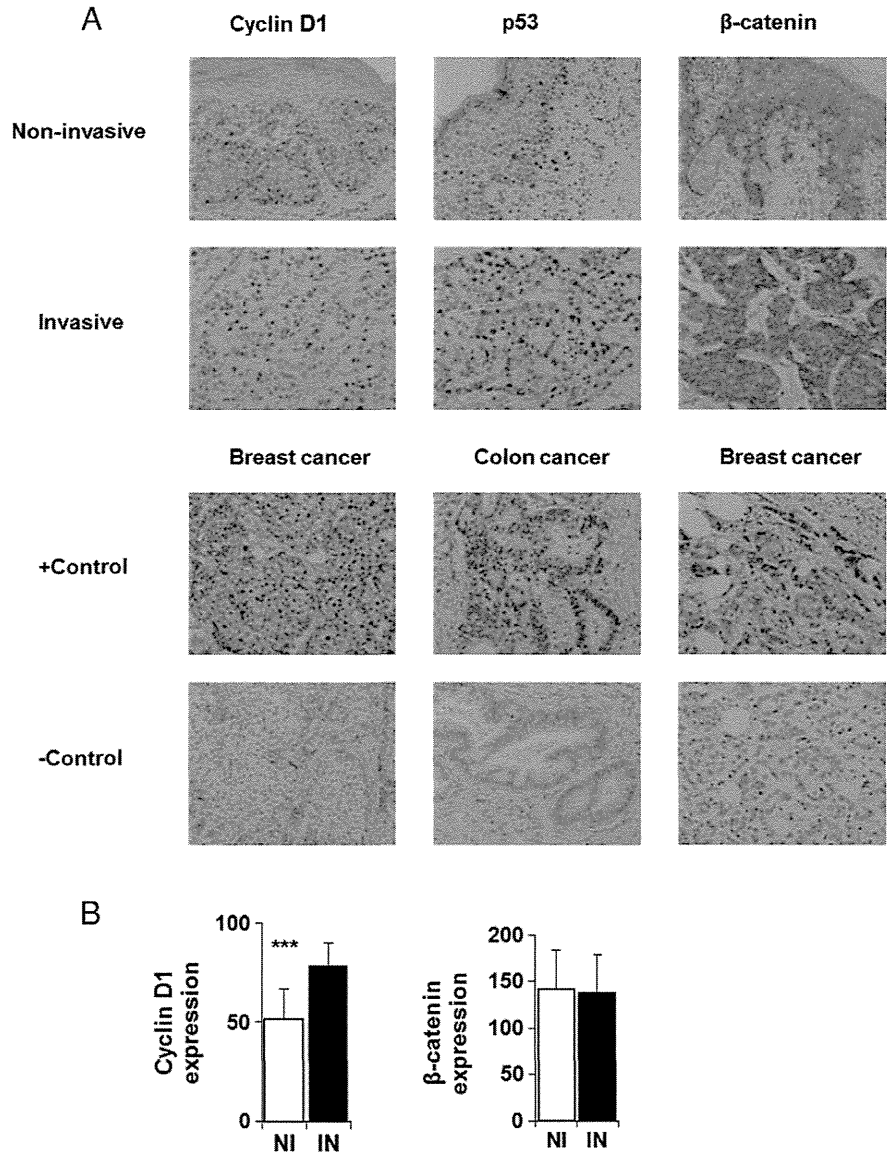

Fig. 4 A, Immunohistochemistry of cyclin D1, p53, and β-catenin in noninvasive and invasive EMPDs. Original magnification ×200. B, Invasive lesion tended to express cyclin D1 significantly higher than noninvasive lesion by LI. +control, positive control; −control, negative control. A significant differences was defined by $***P < .0001$.

interaction of β-catenin with the AR was mediated through a specific protein-protein interaction in prostate cancer [25]. These results above suggest that expression of β-catenin could also play a role in the pathogenesis of EMPD.

FOXP1 has been extensively studied due to its possible roles as a candidate tumor suppressor gene and as an androgen-regulated transcription factor that contributes to the development of prostate cancer [4]. However, little has been known on the status of FOXP1 in EMPD. This is the first study to demonstrate the presence of FOXP1 in both noninvasive and invasive EMPD cases, although no significant differences were detected between invasive and noninvasive phenotypes. FOXP1 expression is also known to be closely related to the degree of malignant biological behavior of epithelial ovarian cancer [26]. However, in breast cancer, FOXP1 gene is commonly detected in both in situ and invasive breast cancer associated with poor survival, indicating that FOXP1 could act as a tumor suppressor [27].

TP53 is the most frequently detected genetic abnormalities of tumor suppressor genes among human malignancies. Alimirah et al [28] reported that p53 negatively regulated the expression of AR in prostate epithelial cells. Results of our present study of p53 in EMPD were consistent with those in the prostate epithelial cells above. p53 status was reported not to demonstrate any association with the invasive properties of EMPD [19]. In addition, p53 overexpression was reported in all the cases of EMPD [29], but its difference between invasive and noninvasive phenotypes has not been reported. In our present study, p53 overexpression was, however, reported in very few EMPD cases, and no differences of the levels or prevalence of p53 were detected between invasive and noninvasive diseases. Further investigations are required to clarify the possible roles of p53 in EMPD.

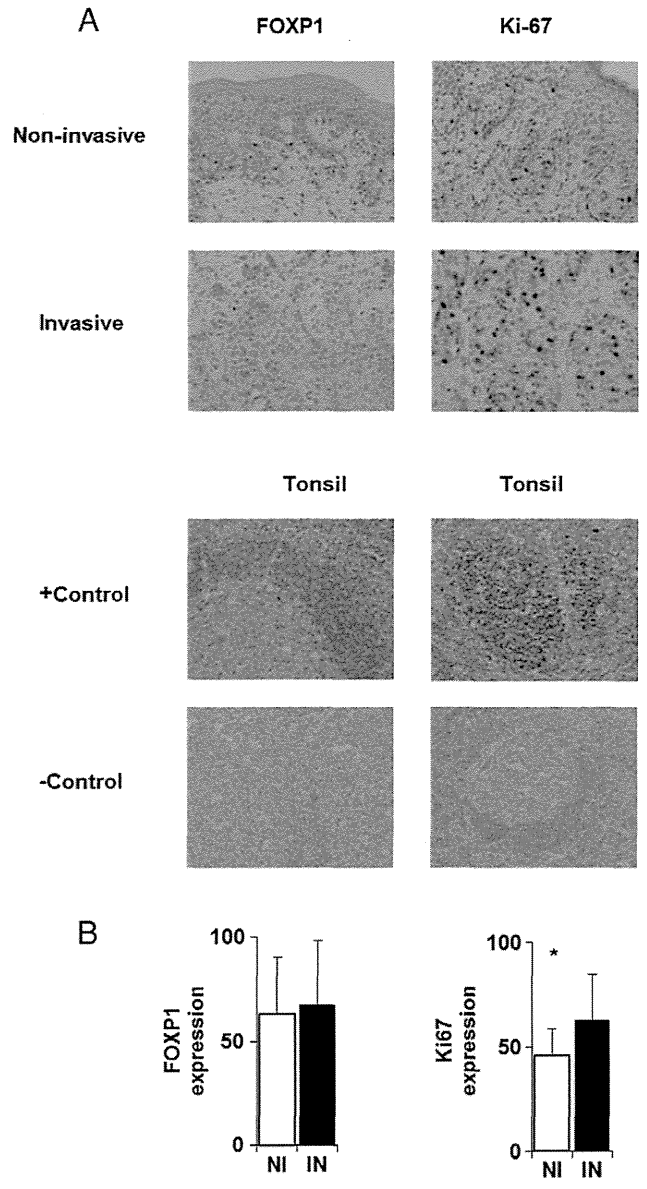

Fig. 5 A, Immunohistochemistry of FOXP1 and Ki-67 in noninvasive and invasive EMPDs. Original magnification ×200. B, Invasive lesion tended to have significantly higher Ki-67 LI than noninvasive lesions. +control, positive control; −control, negative control. A significant differences was defined by $*P < .05$.

In summary, this study firstly demonstrated the importance of the androgen metabolizing/synthesizing enzymes in the local production and action of androgens involved in the invasiveness of Paget cells and cyclin D1 as a potential AR downstream indicator of aggressive or invasive EMPD.

Acknowledgment

The first author receives scholarship support from the Malaysian Government (SLAI) throughout the study period.

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Toxicology in Vitro 29 (2015) 1816–1830

Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

Optimization of the IL-8 Luc assay as an *in vitro* test for skin sensitization

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ARTICLE INFO

Article history:
Received 18 February 2015
Revised 25 June 2015
Accepted 6 July 2015
Available online 15 July 2015

Keywords:
Contact dermatitis
Sensitization test
Luciferase assay
Alternative method
Skin sensitization

ABSTRACT

We previously reported a dataset of the IL-8 Luc assay covering reference chemicals published by ECVAM, in which the effects of chemicals on IL-8 promoter activity were evaluated by an IL-8 reporter cell line, THP-G8 cells. To clarify its performance, we created another dataset of 88 sensitizers and 34 non-sensitizers. Simultaneously, to improve its performance, we changed the incubation time from 5 h to 16 h, deleted the criterion regarding the effects of N-acetylcysteine, and set an exclusion criterion for detergents. These modifications significantly improved its performance. In addition, we examined the following three criteria to judge chemicals as sensitizers: Criterion 1: Fold induction of SLO luciferase activity (FInSLO-LA) $\geqslant$ 1.4, Criterion 2: the lower limit of the 95% confidence interval of FInSLO-LA $\geqslant$ 1.0, Criterion 3: the intersection of criteria 1 and 2. Among them, Criterion 1 produced the best performance, demonstrating that the accuracy, sensitivity and specificity were 81%, 79%, and 90%, respectively. In addition, we found that the IL-8 Luc assay solubilizing chemicals with X-VIVO substantially improved its performance. Finally, the IL-8 Luc assay combined with DPRA and DEREK could improve substantially its performance. These data suggest that the IL-8 Luc assay is a promising test method to screen skin sensitizers.

1. Introduction

In the regulatory context, currently only data from animal experiments are acceptable to assess the skin sensitizing potential of chemicals. The European Union (EU) imposed an animal testing ban effective 2009 on both cosmetics products and their ingredients. This was accompanied by a concomitant marketing ban effective March 1, 2013 if animal tests were conducted after this date for the purpose of cosmetics legislation (Regulation (EC) No. 1223/2009 of the European Parliament and of the Council, 2009). On the other hand, under the European chemicals legislation REACH, skin sensitization data for any chemical registered under the European Chemicals Legislation (REACH, EC 1907/2006) is mandatory, and animal testing should only be performed as a last resort (http://echa.europa.eu/documents/10162/13639/alternatives_test_animals_2014_en.pdf).

Pushed by these ethical and legislative demands, various promising methods have been developed as alternative methods to detect the skin sensitizing potential of chemicals and four methods are currently undergoing formal validation at the European Centre for Validation of Alternative Methods (ECVAM). In 2012, the Organization for Economic Co-operation and Development (OECD) published the adverse outcome pathway (AOP) for skin sensitization (OECD, 2012) in which the key steps in the sensitization process are defined. According to the AOP, 4 methods are considered to target three different steps in the skin sensitization process: protein-binding/haptenization (e.g., the Direct Peptide Reactivity Assay, DPRA) (Gerberick et al., 2004), induction of the Kelch-like ECH-associated protein 1 (Keap-1)/nuclear factor (erythroid-derived 2)-like factor 2 (Nrf2) pathways in keratinocytes (e.g., the KeratinoSens™ assay) (Emter et al., 2010), and the activation of antigen presenting cells such as dendritic cell-like cell lines (The Myeloid U937 Skin Sensitization Test (U-SENS) (Piroird et al., 2015) or the human Cell Line Activation Test, h-CLAT) (Ashikaga et al., 2006; Sakaguchi et al., 2006). However, it is unlikely that a single assay will be sufficient to adequately assess the sensitization potential because of the complexity of the sensitization process (Bauch et al., 2012).

Dendritic cell activation is one of the key steps in sensitization indicated in the AOP for skin sensitization published by the OECD

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http://dx.doi.org/10.1016/j.tiv.2015.07.006
0887-2333/

(2012). In this step, CD54 and CD86 expression is augmented; an increase in interleukin-8 (IL-8) mRNA or IL-8 protein has been suggested as another biomarker for discriminating sensitizers from non-sensitizers in monocyte-derived dendritic cells (MoDCs) (Toebak et al., 2006), U937 cells (Python et al., 2007), or THP-1 cells (Mitjans et al., 2008, 2010; Nukada et al., 2008). IL-8 is well established as a potent chemotactic peptide for neutrophils, T lymphocytes, basophils (Leonard et al., 1990), and NK cells (Sebok et al., 1993). It was recently reported that human immature MoDCs express the IL-8 receptors CXCR1 and CXCR2, which are down-regulated in mature MoDCs (Gouwy et al., 2014) and that accordingly, human immature MoDCs are chemoattracted by IL-8 (Feijoo et al., 2005). It is impossible to demonstrate the exact role of IL-8 in contact hypersensitivity using IL-8 knockout mice because of the lack of a mouse counterpart of IL-8. Regardless, several studies suggest the importance of IL-8 in the DC activation step in the AOP for skin sensitization. Specifically, CCL2 that is coordinately regulated with IL-8 (Singha et al., 2014) plays a crucial role in dendritic cell maturation (Jimenez et al., 2010). On the other hand, Natsuaki et al. have reported DC clusters around macrophages in both the elicitation phase and the sensitization phase in murine contact sensitivity, suggesting the crucial role of CXCR2 expression on DCs in murine contact sensitivity (Natsuaki et al., 2014). Since CXCR2 is a receptor for IL-8 in humans (Marchese et al., 1995; Murphy and Tiffany, 1991), a murine counterpart of IL-8 produced by dermal macrophages may play a crucial role in murine contact sensitization.

In addition, it is now well-recognized that skin sensitization and chemical protein reactivity are linked. Although chemical sensitizers are extremely diverse in molecular weight and structure, most share electrophilic properties and possess intrinsic reactivity toward various amino acids containing nucleophilic heteroatoms (i.e., cysteine, lysine, histidine, arginine, and methionine). Indeed, a correlation between the reactivity of chemicals with cysteine or lysine residues in peptides and their sensitization potential has been demonstrated (Gerberick et al., 2007). Electrophiles can be detected by the Keap1-Nrf2 cellular sensor pathway implicated in the antioxidant response of the cell and recently reviewed by Itoh et al. (2010). Under normal conditions, Keap1 sequesters the transcriptional regulator nuclear Nrf2 in the cytoplasm, provoking its proteasomal degradation. In the presence of electrophiles, the highly reactive cysteine residues of Keap1 are modified, leading to the dissociation of Keap1 from Nrf2. Nrf2 translocates to the nucleus, forms heterodimers with small Maf proteins, and then induces the transcription of genes with an antioxidant response element (ARE) in their promoters (Holland and Fishbein, 2010). These genes code for proteins mostly involved in detoxification, such as heme oxygenase-1 (HO-1) and NADPH-quinone oxidoreductase 1 (Nqo1). Interestingly, Zhang et al. reported that the 5′ flanking region of the IL-8 gene has several areas homologous to the consensus ARE (ATGAC/TnnnGCA/); in addition, Nrf2 caused only a weak induction of IL-8 transcription but significantly increased the half-life of IL-8 mRNA (Zhang et al., 2005). These data suggested that the induction of IL-8 mRNA by haptens is regulated transcriptionally by p38 MAPK and post-transcriptionally by Nrf2. Therefore, the IL-8 Luc assay is a unique screening method for haptens since it detects their effects on p38 MAPK and Keap1-Nrf2.

We established the IL-8 reporter cell assay (IL-8 Luc assay) using a stable THP-1-derived IL-8 reporter cell line, THP-G8, which harbors SLO and SLR luciferase genes under the control of IL-8 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) promoters, respectively (Takahashi et al., 2011). This previous study demonstrated that use of the IL-8 Luc assay to examine 35 chemicals, including reference chemicals published by ECVAM (referred to as the "ECVAM list"; (Casati et al., 2009), resulted in an overall test accuracy of 82%.

In the current study, we increased the number of chemicals for evaluation by the IL-8 Luc assay, explored the reason for false results, modified the procedure and protocol (including the criteria), examined the correlation of parameters between the IL-8 Luc assay and other test methods, and proposed the test battery system.

2. Materials and methods

2.1. Cells and culture

We previously established a reporter cell line, THP-G8 cells derived from the human acute monocytic leukemia cell line THP-1 cells containing stable luciferase orange (SLO) regulated by IL-8 promoter and stable luciferase red (SLR) by GAPDH promoter (Takahashi et al., 2011). THP-G8 cells were cultured in RPMI-1640 (Gibco, Carlsbad, CA) with antibiotic–antimycotic (Invitrogen, Carlsbad, CA) and 10% fetal bovine serum (FBS) (Biological Industries, Kibbutz Beit Haemek, Israel) at 37 °C with 5% CO_2.

2.2. Test chemicals and chemical treatment

We examined 122 chemicals that are listed with their Chemical Abstract Service (CAS) numbers in Table S1. All chemicals had been previously evaluated and classified with the LLNA (Gerberick et al., 2005). Seventy-two sensitizers were evaluated, including 8 extreme, 16 strong, 25 moderate, and 23 weak sensitizers, as classified by the local lymph node assay (LLNA); 28 non-sensitizers were also evaluated, one of which (sodium lauryl sulfate; SLS) was false positive in the LLNA. All the chemicals were purchased from Sigma–Aldrich, St. Louis, MO, at the highest available purity.

Water soluble chemicals were dissolved in distilled water at a concentration of 25 mg/ml, 50 mg/ml, or 100 mg/ml to determine the highest soluble concentration. Chemicals not soluble in water were dissolved in DMSO at 500 mg/ml. If they were not soluble at 500 mg/ml, the highest soluble concentration was determined by diluting the suspension from 500 mg/ml by a factor of two with DMSO. Sonication and vortex mixing were used if needed and the attempt to dissolve the chemical continued for at least 5 min. All dissolved chemicals were used within 4 h of being dissolved in distilled water or DMSO.

To examine the effects of FBS on the IL-8 Luc assay, three methodologies were examined to solubilize haptens: (1) solubilize in DMSO, and then dilute with RPMI-1640 containing 10% FBS, as used in the IL-8 Luc assay (DMSO/FBS); (2) solubilize in DMSO and then dilute with X-VIVO 15 (Lonza, Walkersville, MD) (DMSO/X-VIVO); and (3) solubilize in X-VIVO and then dilute with X-VIVO (X-VIVO/X-VIVO).

For water soluble chemicals, 11 serial dilutions were conducted using RPMI-1640 with 10% FBS diluting by a factor of 2, in the 1st experiment. In the 2nd, 3rd, or 4th experiment, 11 serial dilutions were conducted, diluting by a factor of 1.5. For water insoluble chemicals, 11 serial dilutions were conducted using DMSO as the solvent, diluting by a factor of 2 in the 1st experiment and by a factor of 1.5 in the 2nd, 3rd, and 4th experiments.

Based on the previous report (Saito et al., 2011; Takahashi et al., 2011), THP-G8 cells (5×10^4 cells/50 μl/well) in 96-well black plates (Greiner bio-one GmbH, Frickenhausen, Germany) were cultured for varying time periods. The optimum cell numbers at seeding were based on the previous reports. In some experiments, the cells were pretreated with 25 mM N-acetyl-L-cysteine (NAC) for 30 min.

2.3. IL-8 promoter-luciferase gene reporter assay

The luciferase reporter assay system was constructed using 2 luciferase genes, SLO and SLR, that emit orange and red light, respectively, with a single substrate. The activities of these luciferases can be measured simultaneously and quantitatively with optical filters. This system can rapidly and easily monitor multiple gene expression (Nakajima et al., 2005; Noguchi et al., 2008). In this study, luciferase activity was determined using a microplate-type luminometer with a multi-color detection system, Phelios (Atto Co., Tokyo, Japan), using the Triplu$^{®}$ luciferase assay reagent (TOYOBO Co., Ltd., Osaka, Japan) according to the manufacturer's instructions.

Since some chemicals affected cell viability, we defined the parameter nSLO-LA to represent IL-8 promoter activity. This parameter is calculated by normalizing SLO luciferase activity (SLO-LA) to SLR luciferase activity (SLR-LA). We also calculated the inhibition index of SLR-LA (I.I.-SLR-LA) by dividing the SLR-LA of THP-G8 cells that were treated with chemicals by the SLR-LA of non-treated THP-G8 cells. The fold induction of IL-8 promoter activity (FInSLO-LA) was calculated by dividing the nSLO-LA of THP-G8 cells that were treated with chemicals by that of non-stimulated THP-G8 cells.

We further evaluated the suppressive effect of NAC co-treatment with each chemical by calculation of the inhibition index (I.I.). The I.I. was obtained by dividing the FInSLO-LA of THP-G8 cells stimulated with the chemical in the presence of NAC by the FInSLO-LA stimulated with the chemical alone, using the concentration of chemical at which the chemical induced the largest FInSLO-LA.

The parameters used in the IL-8 Luc assay are shown in Table 1.

Using these parameters, we defined the criteria to identify possible sensitizers. Each criterion is composed of two conditions. Condition 1 defines positive induction or negative induction of SLO-LA in each experiment and condition 2 defines sensitizers or non-sensitizers based on the repeated experiments. In our previous paper (Takahashi et al., 2011), chemicals that demonstrate FInSLO-LA $\geqslant$ 1.4 and I.I. $\leqslant$ 0.8 at the concentration of the chemical at which I.I.-SLR-LA is $\geqslant$0.2 in two or three of three different experiments are categorized as sensitizers and those that do not fulfill these criteria are classified as non-sensitizers. In this study, we optimized the IL-8 Luc assay by examining its performance using six criteria. The precise definition of each criterion is summarized in Table 2. Briefly, these six criteria are different from the original criterion in condition 2: chemicals are categorized as sensitizers when they fulfill condition 1 in two or three of three different experiments in the original criterion, while they are categorized as sensitizers when they fulfill condition 1 in two of two to four different experiments in the new six criteria. Among them, Criterion A, B, and C include the condition I.I. $\leqslant$ 0.8, while Criterion 1, 2, and 3 do not.

Table 1
Parameters used in the IL-8 Luc assay.

Parameters	Description
SLO-LA	SLO luciferase activity regulated by IL-8 promoter
SLR-LA	SLR luciferase activity regulated by G3PDH promoter
nSLO-LA	SLO-LA/SLR-LA
I.I.-SLR-LA	SLR-LA of THP-G8 treated with chemicals/SLR-LA of non-treated THP-G8
FInSLO-LA	nSLO-LA of THP-G8 cells treated with chemicals/nSLO-LA of non-stimulated THP-G8 cells
I.I.	FInSLO-LA of THP-G8 cells stimulated with the chemical and NAC/FInSLO-LA stimulated with the chemical alone

2.4. Real-time monitoring of luciferase activity of THP-G8 cells after chemical stimulation

To clarify the time-dependent change of SLO-LA and SLR-LA of THP-G8 cells after chemical stimulation, THP-G8 was suspended with RPMI1640 supplemented with 10% FBS, 0.1 mM D-luciferin 25 mM HEPES/HCl (pH 7.0), and plated onto 35 mm dish at 2×10^6 cells/dish. After 30 min, 1.6 or 0.8 μg/ml DNCB, 1.6 or 0.8 μg/ml 4-NBB was added to the culture. Bioluminescence was in real-time under a 5% CO_2 atmosphere at 37 °C using the dish-type luminometer AB2500 Kronos (ATTO, Tokyo, Japan).

2.5. Statistics

To demonstrate statistical significance, representative data from at least three independent experiments for each analysis is shown. A one-way ANOVA test followed by Dunnett's post hoc test was used to evaluate statistical significance. p values <0.05 were considered statistically significant. We performed Pearson's product–moment correlation analysis to determine the strength of a correlation.

3. Results

3.1. Determination of the optimal incubation time

The performance of the IL-8 Luc assay, when we first reported (Takahashi et al., 2011), was an accuracy of 86%, a sensitivity of 83%, and a specificity of 90% for 35 chemicals including ECVAM list

Table 2
Criteria used in the IL-8 Luc assay.

Criteria		Condition 1	Condition 2
Original	Sensitizer	FInSLO-LA $\geqslant$ 1.4 and I.I. $\leqslant$ 0.8 at the concentration of the chemical at which I.I.-SLR-LA is $\geqslant$0.2	Fulfill the condition 1 in 2 or 3 of 3 different experiments
Original	Non-sensitizer		Do not fulfill the condition 1 in 2 of 3 different experiments
A	Sensitizer	FInSLO-LA $\geqslant$ 1.4 and I.I. $\leqslant$ 0.8 at the concentration of the chemical at which I.I.-SLR-LA is $\geqslant$0.2	Fulfill the condition 1 in 2 of 2–4 different experiments
B	Sensitizer	FInSLO-LA $\geqslant$ 1.4 and I.I. $\leqslant$ 0.8 at any concentrations	
C	Sensitizer	FInSLO-LA $\geqslant$ 1.4 and I.I. $\leqslant$ 0.8 at the concentration of the chemical at which I.I.-SLR-LA is $\geqslant$0.05	
1	Sensitizer	FInSLO-LA $\geqslant$ 1.4 at the concentration of the chemical at which I.I.-SLR-LA is $\geqslant$0.05	
2	Sensitizer	The lower limit of the 95% confidence interval of FInSLO-LA $\geqslant$ 1.0	
3	Sensitizer	1 and 2	
Either criteria	Non-sensitizer		Do not fulfill the condition 1 in 3 of 3–4 different experiments

(Casati et al., 2009). When we conducted ring trials with 3 different laboratories using 10 coded chemicals, they revealed the accuracy and the intralaboratory reproducibility were not necessarily high enough to conduct further validation studies. Indeed, the accuracy of each laboratory was 80% by Laboratory A, 60% by Laboratory B, and 63% by Laboratory C, respectively, and the intralaboratory reproducibility was 70%. Therefore, we first explored the reason for the poor response of the IL-8 Luc assay to some potent sensitizers, such as DNCB. In our previous study, we examined the time course of FInSLO-LA of THP-G8 cells after LPS stimulation and found the maximum induction of FInSLO-LA between 4 and 7 h after stimulation. In this study, we re-examined the time course of FInSLO-LA after the treatment with strong sensitizers, DNCB and 4-NBB.

We stimulated THP-G8 cells with different concentrations of DNCB or 4-NBB for different time periods and FInSLO-LA was measured (Fig. 1a and b). DNCB and 4-NBB significantly augmented FInSLO-LA dose-dependently from 5 h to 24 h and from 4 h to 24 h after stimulation, respectively. The maximum induction by DNCB was observed between 9 h and 12 h at the concentration of 1.19 μg/ml and 2.67 μg/ml. On the other hand, the maximum induction by 4-NBB was observed between 8 h and 10 h at the concentration of 1.19 μg/ml and at 10 h and 16 h at the concentration of 2.67 μg/ml.

Next, we monitored SLO-LA of THP-G8 cells for 1 min at intervals of 19 min during DNCB or 4-NBB treatment under a dish type luminometer (Fig. 2a and b). Consistent with the results obtained by measuring luciferase activity intermittently, real-time monitoring also demonstrated the increase in FInSLO-LA from 5 h to 24 h by both 0.8 μg/ml and 1.6 μg/ml of DNCB and 4-NBB with maximum induction at 12 h and 10–12 h, respectively.

These results demonstrated that the optimal incubation period with chemicals is around 10 h. This is not practical because one IL-8 Luc assay requires more than 12 h, including general preparation, plating the cells, applying the chemicals and measuring luciferase activity using a luminometer. Therefore, from the practical standpoint, we compared FInSLO-LA between 6 h incubation and 16 h incubation in Figs. 1 and 2. For both DNCB and 4-NBB, intermittent measurement and real-time monitoring indicated that FInSLO-LA at the optimal concentration was much higher in 16 h incubation than in 6 h incubation. This was confirmed by stimulating THP-G8 cells with different concentrations of DNCB and 4-NBB for 6 h and 16 h (data not shown).

Finally, we evaluated the ECVAM list of chemicals by the IL-8 Luc assay with a 16 h incubation period and with the IL-8 Luc assay with a 6 h incubation period and compared the results. As shown in Table 3, most sensitizers increased FInSLO-LA in the 16 h incubation IL-8 Luc assay more than in the 6 h incubation IL-8 Luc assay. In addition, the data from the 16 h incubation period moved isoeugenol from the non-sensitizer category to the sensitizer category, increasing accuracy to 94%.

3.2. Comparison between the criterion with I.I.-SLR-LA ⩾ 0.2 and that with I.I.-SLR-LA ⩾ 0.05

After increasing the incubation time from 6 h to 16 h, we examined 89 chemicals that were used as a data set of h-CLAT (Ashikaga et al., 2010) and evaluated their skin sensitization potential (Table S1). The dataset for the IL-8 Luc assay was created following examination of the IL-8 Luc assay's performance using the six criteria described in Table 2. When we examined its performance using Criterion A, which used the same condition 1 as the original criterion, Cooper statistics of the IL-8 Luc assay for these 89 chemicals yielded an accuracy of 69%, a sensitivity of 59%, and a specificity of 92%, suggesting that the IL-8 Luc assay using the current criterion produce false negative results for a considerable number of sensitizers tested. Closer examination of the data showed that most of the treatments that produced false negative results increased FInSLO-LA more than 1.4 at the concentrations providing I.I.-SLR-LA < 0.2 (data not shown). SLR-LA corresponds with promoter activity of GAPDH gene. GAPDH mRNA is a ubiquitously expressed at moderately abundant levels. It is frequently used as an endogenous control for quantitative real-time polymerase chain reaction because, in some experimental systems, its expression is constant at different times and after various experimental manipulations (Edwards and Denhardt, 1985; Mori et al., 2008; Winer et al., 1999). Indeed, in this study, all but two (benzocaine and methylisothiazolinone) of the 122 chemicals examined did not increase SLR-LA, and most dose-dependently decreased SLR-LA at toxic concentrations, suggesting that SLR-LA can act as an internal control to indicate cell number and viability. Our previous study demonstrated that I.I.-SLR-LA is more sensitive in detecting dying cells than the percentage of PI-excluding cells, and cells showing less than 0.2 of I.I.-SLR-LA retained more than 80% of PI-excluding cells. In this study, we further examined the correlation between the percentage of PI-excluding cells and I.I.-SLR-LA, and confirmed that THP-G8 cells treated with chemicals whose I.I.-SLR-LA showed ⩾0.05 maintained more than 75% of the PI-excluding cells (Fig. 3). We therefore examined the performance of the IL-8 Luc assay using Criterion C that included I.I.-SLR-LA ⩾ 0.05 instead of I.I.-SLR-LA ⩾ 0.2 in the condition 1 and obtained Cooper statistics for these 89 chemicals of 74% accuracy, 69% sensitivity, and 89% specificity.

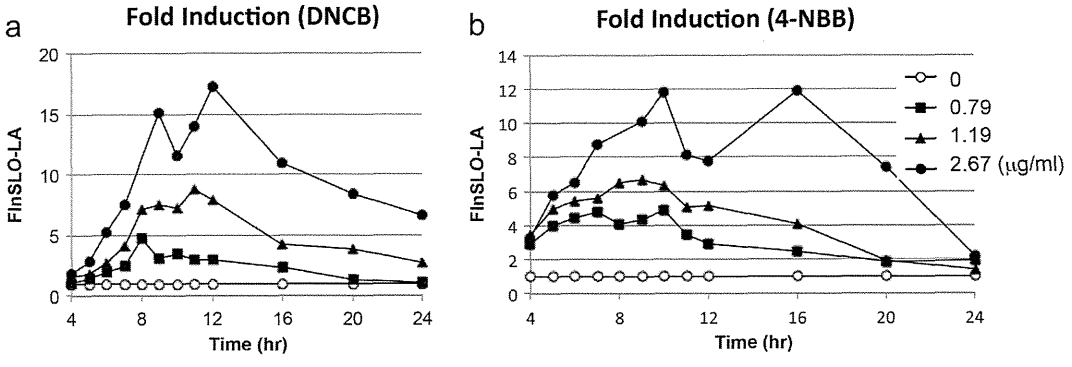

Fig. 1. Determination of the optimal incubation time – time course study for the IL-8 Luc assay. THP-G8 cells were stimulated with the indicated dose of DNCB (a) or 4-NBB (b) for various time periods, and luciferase activity was measured using a microplate-type luminometer.

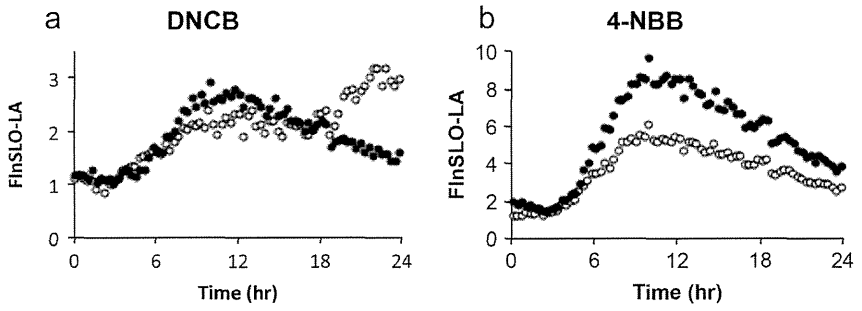

Fig. 2. Determination of the optimal incubation time – real time monitoring of IL-8 luciferase activity. THP-G8 cells were suspended in RPMI-1640 supplemented with 10% FBS, 0.1 mM D-luciferin, 25 mM HEPES/HCl (pH 7.0) and plated onto 35 mm dishes at 2×10^6 cells/dish. After 30 min, 0.8 or 1.6 μg/ml DNCB (a), or 0.8 or 1.6 μg/ml 4-NBB (b), was added to the culture. Bioluminescence was continuously recorded at intervals of 19 min under a 5% CO_2 atmosphere at 37 °C using a dish-type luminometer. Open circles: 0.8 μg/ml, closed circles: 1.6 μg/ml.

3.3. Comparison between the criterion with and without the response to NAC treatment

Despite having accepted the I.I.-SLR-LA ⩾ 0.05 criterion, the IL-8 Luc assay still produced significant numbers of false negative results. Therefore, we further modified the criterion. Although most haptens react with cysteine residues, there may be some exceptions. For example, Gerberick et al. reported that phthalic anhydride and trimellitic anhydride significantly reacted with glutathione and lysine peptides, but not with cysteine peptides in their direct peptide reactivity assay (DPRA) (Gerberick et al., 2007). We hypothesized that removing the condition that I.I. is ⩽0.8 (Criterion 1) would increase the accuracy and specificity of the IL-8 Luc assay. Indeed, this modification significantly improved the performance of the IL-8 Luc assay and significantly improved accuracy and sensitivity (i.e., accuracy of 78% and sensitivity of 77%) but decreased the specificity to 74% (Table S1 and Table 4), when we examined the 122 chemicals (9 extreme, 17 strong, 34 moderate, and 28 weak sensitizers, and 34 non-sensitizers as classified by the LLNA (Gerberick et al., 2005)) as a dataset for h-CLAT (Ashikaga et al., 2010; Nukada et al., 2012; Takenouchi et al., 2013).

3.4. Comparison between the criterion with FInSLO-LA ⩾ 1.4, the criterion with the lower limit of the 95% confidence interval of FInSLO-LA ⩾ 1.0, and their combination

We empirically determined the condition of FInSLO-LA ⩾ 1.4 (Takahashi et al., 2011), similar to the condition used in h-CLAT (Sakaguchi et al., 2006). In contrast, each independent repetition was statistically evaluated in the KeratinoSens assay (Emter et al., 2010). We likewise tried to evaluate each IL-8 Luc assay experiment statistically. When we evaluated 122 chemicals using the criterion of the lower limit of the 95% confidence interval of FInSLO-LA ⩾ 1.0 (Criterion 2) did not necessarily improve the performance of the IL-8 Luc assay (accuracy of 74%, sensitivity of 80%, and 59% of specificity). We also examined the performance of the combination of Criterion 1 and Criterion 2 (Criterion 3). Both the accuracy and sensitivity of Criterion 3 was inferior to those of Criterion 1, although the specificity of Criterion 3 and Criterion 1 was equal (Table S1 and Table 4).

3.5. The factors that produce false negative or positive results in the IL-8 Luc assay (1) – physical properties

The IL-8 Luc assay conducted according to Criterion 1 produced 9 false negative results among 88 haptens determined by LLNA. To clarify the underlying reason for these false negative results, we first compared two physical properties of haptens judged by the IL-8 Luc assay to be sensitizers (true positive) and those judged to be non-sensitizers (false negative): molecular weight, and Log Ko/*w* or water solubility. The results could not demonstrate statistically significant differences (Fig. 4). Indeed, 8 sensitizers had Log Ko/*w* values above 3.5. The accuracy and sensitivity of these chemicals by the IL-8 Luc assay were both 87.5%.

3.6. The factors that produce false negative or positive results in IL-8 Luc assay (2) – the effects of FBS

The effects of FBS in the culture medium were considered next. Recently, several researchers have demonstrated that most sensitizers can bind to both FBS and cellular proteins, although the distribution of covalent binding to cellular or FBS protein varies depending on the hapten (Divkovic et al., 2005; Hopkins et al., 2005; Saito et al., 2013). These reports suggested that the amount of reactive electrophiles in haptens to bind to nucleophiles of cellular protein might be reduced in the presence of FBS.

Therefore, we examined whether a reduction in FBS concentration in the IL-8 Luc assay can reduce false negative results. First, THP-G8 cells were stimulated with oxazolone, which was judged as a non-sensitizer by the IL-8 Luc assay. Three methodologies were examined to solubilize oxazolone: (1) solubilize in DMSO, and then dilute with RPMI-1640 containing 10% FBS, as used in the IL-8 Luc assay (DMSO/FBS); (2) solubilize in DMSO and then dilute with X-VIVO (DMSO/X-VIVO); and (3) solubilize in X-VIVO and then dilute with X-VIVO (X-VIVO/X-VIVO) (Fig. 5a–c). As we have repeatedly demonstrated, oxazolone diluted with DMSO/FBS did not induce significant induction of FInSLO-LA at the concentration at which I.I.-SLR-LA showed ⩾0.05. In contrast, oxazolone diluted with X-VIVO/X-VIVO significantly and dose-dependently induced FInSLO-LA at the concentration at which I.I.-SLR-LA showed ⩾0.10. Oxazolone diluted with DMSO/X-VIVO significantly induced FInSLO-LA at the concentration at which I.I.-SLR-LA showed ⩾0.6, but significant induction was observed only at this single concentration. These results clearly demonstrated that the dilution of oxazolone with X-VIVO significantly improved the response of THP-G8 cells. Furthermore, contrary to our expectation, even solubilization of oxazolone with DMSO did not necessarily improve THP-G8 response.

Clearly, FBS perturbed the response of THP-G8 cells for oxazolone; we therefore next examined whether changing the culture medium during treatment with the chemicals from RPMI-1640 with 10% FBS to X-VIVO improved the response of THP-G8 cells to haptens. The results clearly showed that the response of THP-G8 cells to oxazolone became far weaker in X-VIVO than that RPMI-1640 with 10% FBS (data not shown).

Table 3
Comparison of the performance between IL-8 Luc assay (16 h) and IL-8 Luc assay (6 h).

Chemical	LLNA	The IL-8 Luc assay (6 h) Takahashi et al. in Toxicol. Sci.								The IL-8 Luc assay (16 h)							
		1st		2nd		3rd		Positive exp	Decision	1st		2nd		3rd		Positive exp	Decision
		FInSLO-LA	I.I.	FInSLO-LA	I.I.	FInSLO-LA	I.I.			FInSLO-LA	I.I.	FInSLO-LA	I.I.	FInSLO-LA	I.I.		
Oxazolone	Sensitizer	1	N.D.	1	N.D.			0	Non-sens	1.43	1.15	1.29	1.03			0	Non-sens
4-NBB	Sensitizer	3.9	0.27	4.4	0.21			2	Sens	7.4	0.14	6.63	0.14			2	Sens
DNCB	Sensitizer	2.3	0.44	1.8	0.53			2	Sens	5.83	0.15	10.38	0.08			2	Sens
MDGN	Sensitizer	1.6	0.65	2.7	0.5			2	Sens	3.68	0.28	1.87	0.47			2	Sens
Glyoxal	Sensitizer	1.7	0.77	1.4	0.76			2	Sens	2.97	0.71	3.76	0.3			2	Sens
2-MBT	Sensitizer	1.9	0.7	1.9	0.76			2	Sens	2.29	0.78	6.36	0.56			2	Sens
Cinnamal	Sensitizer	2	0.54	2.7	0.42			2	Sens	5.09	0.19	8.05	0.15			2	Sens
TMTD	Sensitizer	1.7	0.63	3.6	0.44			2	Sens	7.28	0.14	3.95	0.28			2	Sens
PPD	Pre/pro hapten	1.5	0.66	2.3	0.57			2	Sens	1.61	0.61	1.76	0.6			2	Sens
Isoeugenol	Pre/pro hapten	1.8	0.79	1.8	0.86	1.55	1.04	1	Non-sens	2.94	0.74	4.07	0.44			2	Sens
Eugenol	Pre/pro hapten	2.1	0.64	1.7	0.77			2	Sens	4.85	0.69	2.72	1.17	1.69	0.72	2	Sens
Cinnamic alcohol	Pre/pro hapten	2.1	0.47	2.3	0.76			2	Sens	7.68	0.54	8.52	0.54			2	Sens
Glycerol	Irritant	1.5	0.82	1.4	0.83			0	Non-sens	1.12	0.95	1.03	1.25			0	Non-sens
Salicylic acid	Irritant	1.3	1.27	1.1	0.98			0	Non-sens	1	1	1	1			0	Non-sens
Lactic acid	Irritant	1.2	0.82	1.1	1.32			0	Non-sens	1.35	1.95	1.54	2.18			0	Non-sens
SLS	Irritant	3.1	0.88	4.6	0.86			0	Non-sens	2.84	1.18	4.49	1.04			0	Non-sens

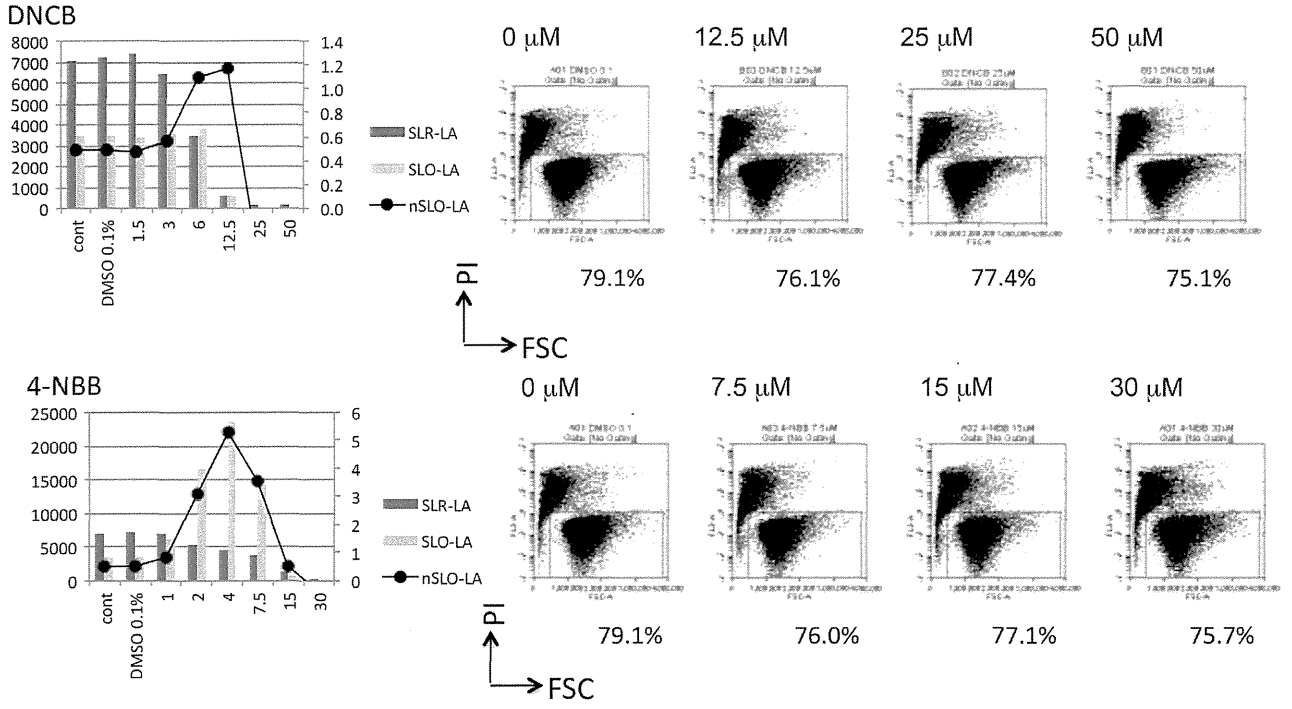

Fig. 3. Comparison between criterion with I.I.-SLR-LA ⩾ 0.2 and that with I.I.-SLR-LA ⩾ 0.05. Cell viability was determined by a PI exclusion assay using flow cytometry. THP-G8 cells were stimulated with the indicated dose of DNCB or 4-NBB. In this PI exclusion assay, THP-G8 cells after chemical treatment were mixed with 30 μg/ml of PI, and the live cells (which are not permeable to PI) were counted using flow cytometry. The results of the luciferase assay are shown on the left.

Table 4
Comparison of the performance among different criterion.

Criteria	With I.I. criterion (I.I. ⩽ 0.8)			Without I.I. criterion		
	Without I.I.-SLR-LA criterion	*I.I.-SLR-LA ⩾ 0.2*	*I.I.-SLR-LA ⩾ 0.05*	*Criterion 1*	*Criterion 2*	*Criterion 3*
Chemical numbers	98	89	97	122	122	122
Accuracy	0.75	0.69	0.74	0.77	0.73	0.73
Sensitivity	0.69	0.59	0.69	0.77	0.80	0.72
Specificity	0.89	0.92	0.89	0.74	0.59	0.74

Next, we examined whether the other chemicals providing false negative results in the IL-8 Luc assay could be judged as sensitizers if diluted with X-VIVO/X-VIVO (Table 5). Interestingly, 5 of the 14 chemicals that showed false negative results were judged as sensitizers. To confirm the efficacy of solubilization of chemicals with X-VIVO, we re-evaluated the chemicals in the ECVAM list (Table 6). The IL-8 Luc assay using chemicals diluted with X-VIVO/X-VIVO increased the FInSLO-LA of all sensitizers, i.e., oxazolone, 4-NBB, DNCB, MDGN, eugenol, and PPD, and changed the judgment of oxazolone. As a result, the Cooper statistics of the IL-8 Luc assay using chemicals diluted with X-VIVO yielded an accuracy of 94%, a sensitivity of 100%, and a specificity of 75%. These data suggested that the IL-8 Luc assay diluted with X-VIVO can improve the accuracy and sensitivity, while it does not lower the specificity.

3.7. The factors that produce false negative or positive results in IL-8 Luc assay (3) – detergents

The IL-8 Luc assay conducted according to Criterion 1 produced 8 false positive results among 28 non-sensitizers, of which hexadecyltrimethylammonium bromide, benzalkonium chloride, Tween-80, and SLS are well-known detergents. It has been reported that the treatment of reconstructed human epidermis with detergents, such as sodium lauryl sulfate, triton, and benzalkonium chloride, increased IL-8 mRNA levels in and IL-8 release from the cells (Coquette et al., 1999). Moreover, White et al. have demonstrated that SLS induced early growth response-1 (EGR-1) depending on the activation of MEK1/p44/42 ERK and EGFR (White et al., 2011). In contrast to these observations on epidermal cells, several researchers examined IL-8 release by THP-1 cells and demonstrated the lack of IL-8 production when stimulated with SLS (Mitjans et al., 2008; Trompezinski et al., 2008). However, the concentration of SLS used in these studies was 30 μg/ml or less. Since the optimal concentration of SLS to induce FInSLO-LA is 50 μg/ml, and the concentration range that significantly augmented FInSLO-LA was narrow, the previous authors either did not stimulate THP-1 with the optimal concentration of SLS or there was a discrepancy between IL-8 release and IL-8 mRNA induction that corresponds with FInSLO-LA. Regardless, we propose not to evaluate detergents by the IL-8 Luc assay.

3.8. The factors that produce false negative or positive results in the IL-8 Luc assay (4) – relative human skin sensitizing potency

Recently, Basketter et al. (2014) collected data regarding the sensitizing potential of chemicals to human skin and classified 131 chemicals into 6 categories based on their relative human skin

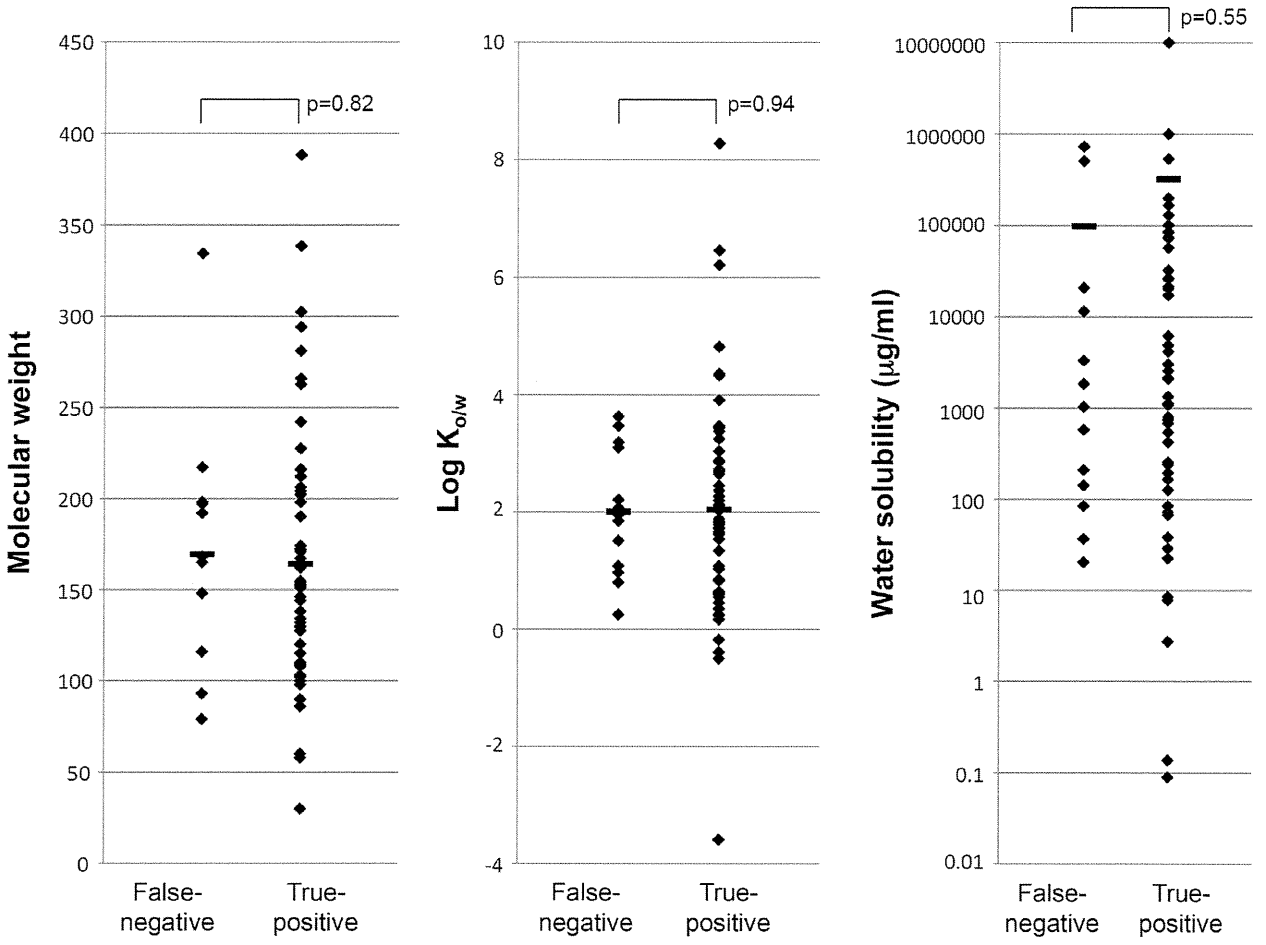

Fig. 4. Factors responsible for false negative or positive results in the IL-8 Luc assay (chemical properties). Sensitizers judged by the LLNA are divided into false negative and true positive groups according to the results of the IL-8 Luc assay. Molecular weight, Log Ko/*w*, and water solubility of each chemical are plotted on the *y*-axis for each group. The mean and *p* value compared with Student's *t*-test are shown.

sensitizing potency, with category 1 being the most potent, category 5 being the least potent, and category 6 being true non-sensitizers. Comparison of the relative human skin sensitizing potency of these chemicals with their LLNA evaluation provided excellent correlation in that all sensitizers judged by relative human skin sensitizing potency are also classified as sensitizers by LLNA. However, there were some differences between the two categorization schemes. For example, although vanillin and benzalkonium chloride are classified as non-sensitizers by LLNA, relative human skin sensitizing potency included them in category 5, the least potent sensitizer. The IL-8 Luc assay also judged vanillin as sensitizers.

3.9. The performance of the IL-8 Luc assay after considering the exclusion criterion and human sensitization potential, and using X-VIVO as a solvent

If we delete the data of hexadecyltrimethylammonium bromide, Tween-80, SLS and benzalkonium chloride and consider vanillin as a sensitizer, the performance of the IL-8 Luc assay was accuracy of 81%, sensitivity of 79%, and specificity of 90% in Criterion 1, accuracy of 77%, sensitivity of 80%, and specificity of 69% in Criterion 2, and accuracy of 77%, sensitivity of 73%, and specificity of 90 in Criterion 3 (Table 7). Furthermore, if we consider the results using X-VIVO as a solvent, the performance of the IL-8 Luc assay was accuracy of 90%, sensitivity of 90%, and specificity of 90% in Criterion 1, accuracy of 82%, sensitivity of 87%, and specificity of 69% in Criterion 2, and accuracy of 86%, sensitivity of 84%, and specificity of 90% in Criterion 3 (Table 8).

3.10. Correlation between the IL-8 Luc assay and other screening methods

Next, we examined the correlation of the parameters between the IL-8 Luc assay and other assays. Statistical analysis of the correlation between FInSLO-LA, and the percent depletion of peptides containing either lysine or cysteine in DPRA, provided no significant correlation (Fig. 6). Similarly, we also examined the correlation between the minimum concentration required to induce more than 1.4 of FInSLO-LA in the IL-8 Luc assay (IL-8 Luc assay minimum induction threshold (MIT)) and that required to induce more than 150% of CD86 augmentation (h-CLAT MIT (CD86 EC150)) (Fig. 7a) or more than 200% of CD54 in h-CLAT (h-CLAT MIT (CD54 EC200)) (Fig. 7b). The results demonstrated weak correlation between them (Fig. 7a and b). In contrast, there was no significant correlation between the IL-8 Luc assay MIT and EC 1.5 of KeratinoSens (Fig. 7c).

3.11. Score-based battery system

To improve the performance of the IL-8 Luc assay for predicting sensitizing potential and the potency of chemicals, we developed a

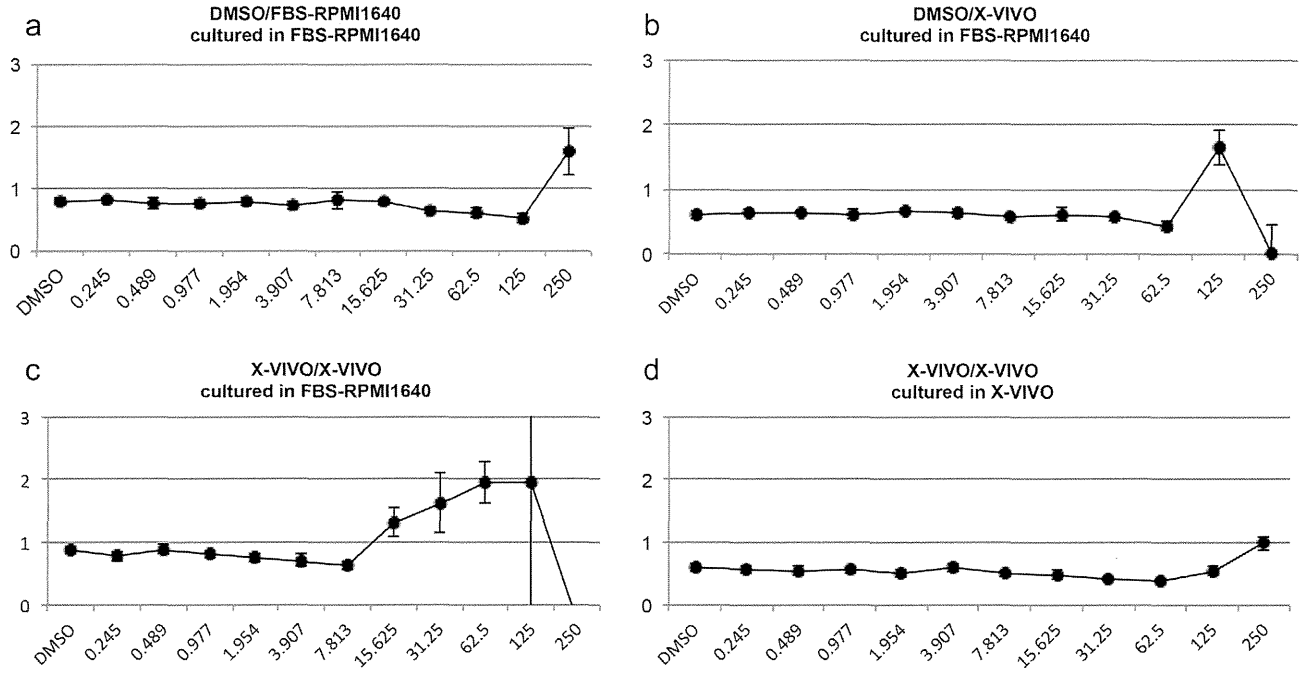

Fig. 5. The effects of FBS on the IL-8 Luc assay. To stimulate THP-G8 cells, we solubilized oxazolone in the following 3 ways: (a) solubilize in DMSO and then dilute with RPMI-1640 containing 10% FBS as used in the IL-8 Luc assay (DMSO/FBS); (b) solubilize in DMSO and then dilute with X-VIVO (DMSO/X-VIVO); and (c) solubilize in X-VIVO and then dilute with X-VIVO (X-VIVO/X-VIVO).

Table 5
Re-evaluation of chemicals demonstrating false negative results by the IL-8 Luc assay using X-VIVO as a solvent.

Chemicals	Experiments		Judgment
	1st	2nd	
Oxazolone	1.66	3.98	Sensitizer
Phthalic anhydride	1.09	1.60	
2-Hydroxyethyl acrylate	1.00	1.00	
Ethylenediamine	1.96	2.38	Sensitizer
Methyl-2-nonynoate	1.93	5.91	Sensitizer
3,4-Dihydrocoumarin	1.00	1.00	
Trimellitic anhydride	2.28	1.66	Sensitizer
1-Bromohexxane	1.07	1.05	
4-Allylanisole	4.67	19.71	Sensitizer
Benzocaine	1.11	1.00	
Ethyleneglycol dimethacrylate	4.98	4.36	Sensitizer
Penicillin G	1.35	1.00	
Pyridine	1.09	1.00	
Aniline	1.03	1.12	

test battery by assigning scores to the outcomes in each single test, based on the concept reported by Jowsey et al. (2006). We converted the results of the IL-8 Luc assay and DPRA into a score from 0 to 2 based on the sensitizing potency classification. In the IL-8 Luc assay, we first obtained the maximum value of FInSLO-LA (MAX FInSLO-LA) and the MIT for each chemical. MAX FInSLO-LA was the largest value of FInSLO-LA in all repeated experiments. The MIT was defined as the lowest value among the concentrations in all repeated experiments in which the chemical induced FInSLO-LA more than 1.4. MAX FInSLO-LA/MIT was calculated by dividing MAX FInSLO-LA by MIT and each chemical was given a score of 0, 1, or 2 based on the criterion shown in Table 9.

The mean ± SEM of MAX FInSLO-LA/MIT of chemicals in each group with different allergenicity was shown in Fig. 8a. Generally, the MAX FInSLO-LA/MIT values were higher in the group of chemicals containing more potent sensitizers. The data and the scoring system published by Nukada et al. (2013) and Jaworska et al. (2013) were used for DPRA and DEREK. The positive results in DPRA were classified as strong if the average depletion score of cysteine and lysine peptide was above 22.62% and weak if it was above 6.376% and less than or equal to 22.62%. Similarly, if the analysis outcome by DEREK was defined as probable or plausible, the test chemical was judged as a sensitizer. If defined as doubtful or no report, the test chemical was judged as a non-sensitizer. For the 103 test chemicals, the total battery score between 0 and 5 was then calculated by the sum of the individual scores. The box plot indicated the resulting scores split up for the five LLNA potency classifications (i.e., extreme, strong, moderate, weak, and not classified) (Fig. 8b). The median values in the box plot of the IL-8 Luc assay combined with DPRA and DEREK decreased with the corresponding LLNA potency classes with a better linear correlation ($R^2 = 0.90$) than that of the IL-8 Luc assay alone.

Table 6
Re-evaluation of the chemicals in the ECVAM List by the IL-8 Luc assay using X-VIVO as a solvent.

Chemicals	Experiments		Judgment
	1st	2nd	
Oxazolone	7.69	3.98	Sensitizer
4-NBB	10.03	8.77	Sensitizer
Glyoxal	3.50	2.23	Sensitizer
2-MBT	5.17	4.37	Sensitizer
DNCB	14.54	9.48	Sensitizer
MDGN	4.23	4.35	Sensitizer
Cinnamal	1.53	5.57	Sensitizer
TMTD	4.58	3.61	Sensitizer
PPD	4.30	2.48	Sensitizer
Isoeugenol	1.89	1.59	Sensitizer
Eugenol	2.57	2.52	Sensitizer
Cinnamic alcohol	7.24	7.13	Sensitizer
Glycerol	1.09	1.04	Non-sensitizer
Salicylic acid	1.18	1.00	Non-sensitizer
Lactic acid	1.00	1.08	Non-sensitizer
SLS	2.94	2.88	Sensitizer

Table 7
Performance of the IL-8 Luc assay after deleting the data for detergents and considering human sensitizing potential.

Criteria	IL-8 Luc assay			Modified IL-8 Luc assay		
	Criterion 1	Criterion 2	Criterion 3	Criterion 1	Criterion 2	Criterion 3
Chemical numbers	122	122	122	118	118	118
Accuracy	0.77	0.73	0.73	0.81	0.77	0.77
Sensitivity	0.77	0.80	0.72	0.79	0.80	0.73
Specificity	0.74	0.59	0.74	0.90	0.69	0.90

Modified IL-8 Luc assay: Deleting the data for detergents and considering human sensitizing potential.

Table 8
Performance of the IL-8 Luc assay after deleting the data for detergents, considering human sensitizing potential, and changing to X-VIVO as a solvent.

Criteria	IL-8 Luc assay			Modified IL-8 Luc assay		
	Criterion 1	Criterion 2	Criterion 3	Criterion 1	Criterion 2	Criterion 3
Chemical numbers	122	122	122	118	118	118
Accuracy	0.77	0.73	0.73	0.90	0.82	0.86
Sensitivity	0.77	0.80	0.72	0.90	0.87	0.84
Specificity	0.74	0.59	0.74	0.90	0.69	0.90

Modified IL-8 Luc assay: Deleting the data for detergents, considering human sensitizing potential, and changing to X-VIVO as a solvent.

Next, we set the positive criterion as a score of above 2 for hazard identification. Furthermore, to determine the sensitizing potency using a three-rank classification (strong, weak, and not classified) from the total battery scores, we set the strong score as 5, the weak score as 4, 3, and 2, and not classified as 1 and 0. Table 10 demonstrates the results of hazard identification and shows that the sensitivity, the specificity and the overall accuracy of the score-based battery system was 87.1%, 80.8%, and 85.4%, respectively, according to the Cooper statistics. All these parameters were improved compared to either DPRA or the IL-8 Luc assay alone for the examined chemical sets.

In the potency classification (Table 11), the strong class in this battery system included 14 of 23 extreme and strong sensitizers in the LLNA. Likewise, the weak class included 38 of 54 moderate and weak sensitizers in the LLNA. Thus, the accuracy in the potency prediction with the battery system was 70.9%, the over-prediction rate was 10.7%, and the under-prediction rate was 18.4%. These data suggested that the potential and potency of sensitizing chemicals were identified with good reliability by the score-based battery system.

3.12. Tiered system with h-CLAT and DPRA

We next developed a tiered approach, weighing the predictive performance of the IL-8 Luc assay and DPRA for the 103 evaluated chemicals. The IL-8 Luc assay provided high sensitivity and sufficiently detected extreme and strong sensitizers (Fig. 8a), indicating that the IL-8 Luc assay was a good first step for a tiered approach. The positive results in the IL-8 Luc assay were classified into two classes, strong or weak, based on Max-FInSLO-LA/MIT values. Then, to reliably predict weak and moderate sensitizers, which the IL-8 Luc assay failed to detect, we determined that DPRA was a good second step. The positive results in DPRA were classified into the weak class regardless of the average depletion score. If the chemical scored as negative in both tests, it was considered as not classified. As shown in Table 12, the tiered system provided a relatively high sensitivity of 96.1% (74 of 77 sensitizers) and an accuracy of 87.4% (90 of 103 test chemicals).

The potency classification by the tiered system is summarized in Table 13. All tested chemicals, except for 1 chemical classified as strong in the tiered system, were sensitizers in the LLNA, suggesting a positive predictivity of 98.0% in this class. Moreover, 16 of 19 chemicals categorized as not classified by the tiered system were non-sensitizers in the LLNA, suggesting a negative predictivity of 84.2%. The false negative rate in the weak class was 5.5% (3 of 54 sensitizers). Importantly, the tiered system with the IL-8 Luc assay and DPRA could detect 51 of 54 sensitizers classified as moderate and weak in the LLNA. The strong, weak, and non-classified classes have good correlation with the extreme/strong, moderate/weak, and non-classified class in the LLNA. Thus, the accuracy of potency prediction with the battery system was 62.1%, the over-prediction rate was 28.2%, and the under-prediction rate was 9.7%.

4. Discussion

In parallel with conducting the inter- and intra-laboratory reproducibility tests, we tried to improve the performance of the IL-8 Luc assay. Consequently, we found that the modification of the original protocol of the IL-8 Luc assay by increasing the incubation time, revising the lower limit of I.I.-SLR-LA, deleting the process to examine the effects of NAC, and setting the exclusion criterion could substantially improve the performance of the IL-8 Luc assay. In final, by taking these modifications into account, the performance of the IL-8 Luc assay was accuracy of 81%, sensitivity of 79%, and specificity of 90% in the evaluation of 122 chemicals.

In addition, we here examined the difference in determining positive induction of SLO-LA between Criterion 1 and Criterion 2. Condition 1 used in Criterion 1 was determined empirically, while that used in Criterion 2 was based on the statistical significance. After examining the 122 chemicals, the concordance rate was 90%, which suggests that condition 1 used in Criterion 1, FInSLO-LA $\geqslant$ 1.4 at the concentration of the chemical at which I.I.-SLR-LA is $\geqslant$0.05, is statistically supported. We also accepted the new condition 2 to judge sensitizers based on the repeated experiments: chemicals are categorized as sensitizers when they fulfill condition 1 in two of two to four different experiments, while they were categorized as sensitizers when they fulfilled condition 1 in two or three of three different experiments in our previous report (Takahashi et al., 2011). In this new condition 2, some experiments showing positive induction are repeated only twice to judge sensitizers, while those showing negative induction are repeated three times to judge non-sensitizers. Therefore, to verify this condition, we repeated three experiments for 21 chemicals

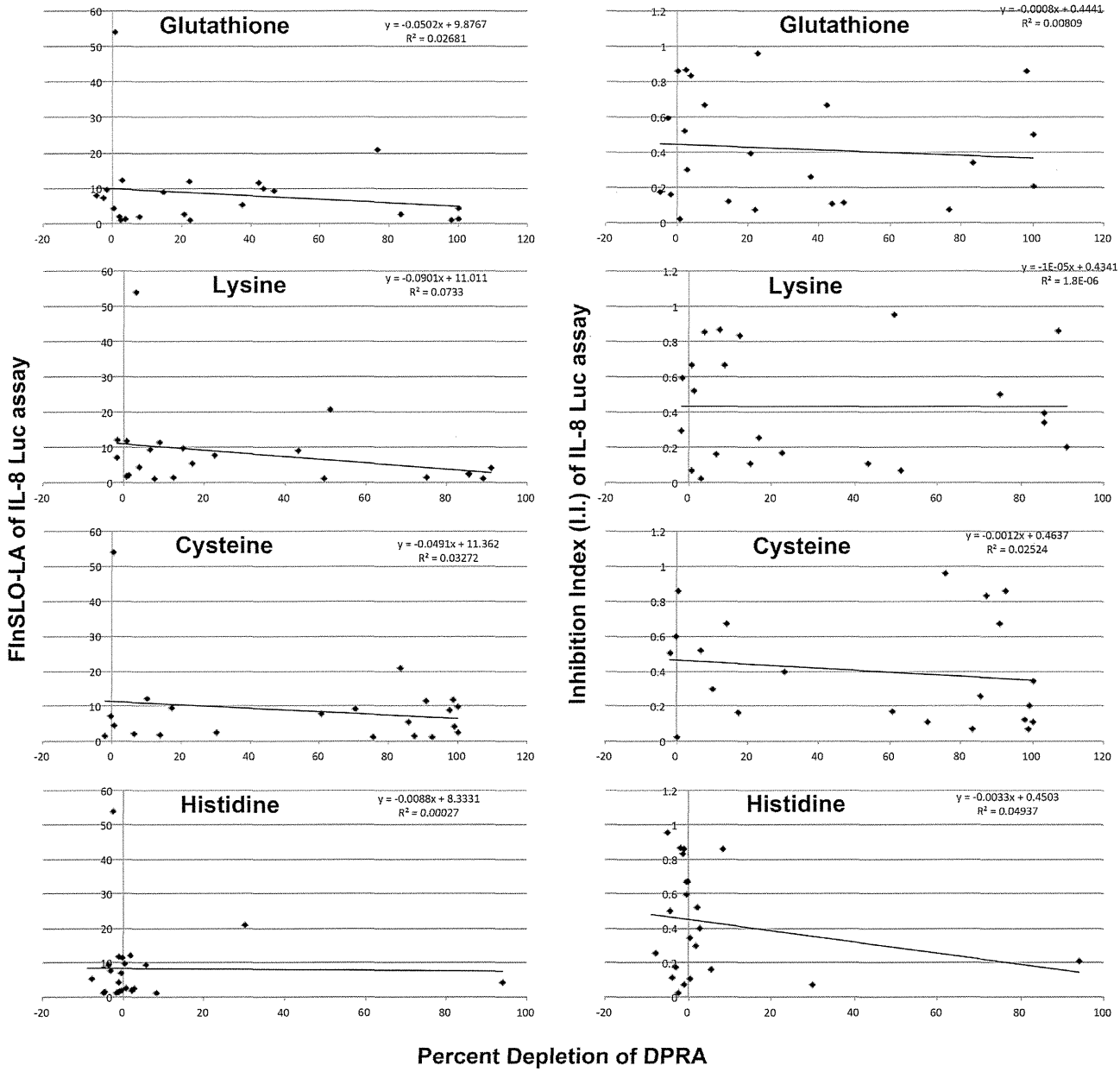

Fig. 6. Correlation between the IL-8 Luc assay and other screening methods (DPRA). The correlation between FInSLO-LA and the percent depletion of peptides containing either lysine or cysteine in DPRA was examined.

that showed positive induction in the first two experiments. The results demonstrated that the third experiment also showed positive induction in all 21 chemicals, verifying the new condition 2.

In this study, we examined 4 detergents, i.e., hexadecyltrimethylammonium bromide, benzalkonium chloride, Tween-80 and SLS and found that these detergents significantly increased FInSLO-LA, which suggested that they could stimulate IL-8 promoter activity. These data are consistent with the previous reports that have demonstrated IL-8 production by keratinocytes after the stimulation of various detergents (Coquette et al., 1999; White et al., 2011). Detergents can be categorized according to the charge present in the hydrophilic head (after dissociation in aqueous solution) into four primary groups: anionic, cationic, amphoteric (dual charge) and nonionic (Corazza et al., 2010). Quaternary ammonium compounds, such as hexadecyltrimethylammonium bromide and benzalkonium chloride, are cationic, while Tween-80 and SLS are nonionic and anionic, respectively. Therefore, our data suggested that detergents could stimulate IL-8 promoter activity irrespective of kinds of the charges present in the hydrophilic head. It is not necessarily clear how detergents induce IL-8 mRNA. In contrast to haptens that induce IL-8 mRNA expression by DCs or THP-1 cells depending on p38 MAPK, however, at least SLS has been demonstrated to induce IL-8 mRNA depending on the activation of MEK1/p44/42 ERK (White et al., 2011). Indeed, our previous study demonstrated NAC could not suppress FInSLO-LA induced by Tween-80, SLS, and benzalkonium chloride, while it significantly attenuated it induced by most haptens (Takahashi et al., 2011). Therefore, it is plausible to include the criterion for examining the effects of NAC on FInSLO-LA. However, since the IL-8 Luc assay including the criterion to examine the effects of NAC makes the test