Construction of a Three-Dimensional Human Skin Model Consisting of Keratinocytes, Dendritic Cells and Fibroblasts and Application of This Model for Alternative Animal Testing of Immune-Sensitizing Compounds*

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In order to establish *in vitro* evaluation of the sensitization of human skin, we attempted to make a three-dimensional human skin model consisting of three different cells, dendritic cells, keratinocytes and fibroblasts. The viability of the cells in the human skin model was observed after staining with hematoxylin and eosin. After 11–14-day incubation (horny layer was initially observed), the three-dimensional human skin model was used for experiments. Due to 2,4-dinitrochlorobenzene (DNCB) under a non-cytotoxic dose, the keratinocytes and dendritic cells in the human skin model significantly induced IL-4 release into the incubating medium and dendritic cells induced CD86 expression. On the other hand, with sodium dodecyl sulfate (SDS; non-sensitizer), the keratinocytes and dendritic cells did not significantly induce IL-4 release and the dendritic cells did not induce CD86 expression. The results suggested that this three-dimensional human skin model with dendritic cells could be applied as an alternative to animal testing of immune-sensitizing compounds.

Key words : three-dimensional human skin model, CD86, dendritic cells, keratinocytes, fibroblasts, immune-sensitizing compounds, DNCB, IL-4, *in vitro* evaluation, cytotoxicity

1. Introduction

For the safety of cosmetic ingredients, immune-sensitizing is important. Unfortunately, these are no *in vitro* human skin models applicable to the evaluation of immune-sensitizing compounds. Recently, to study an *in vitro* evaluation method of immune-sensitizing compounds, normal human dendrite cells were used. De Smedt et al.¹⁾ reported that after 24-h exposure of normal human dendrite cells to NiCl₂, the CD86+ expression on cells and IL-6 production should significantly increase, but there were no reactions in the case of sodium dodecyl sulfate (SDS). Furue et al.²⁾ reported that keratinocytes produce various kinds of cytokines (IL-1, TNF- α , GM-CSF, etc.) and initiated survival and functional maturation of dendritic cells. Yoshida et al.³⁾ reported that after 24-h exposure of the human monocytic leukemia cell line (THP-1) for h-CLAT (human Cell Line Activation Test) method to 2,4-dinitrochlorobenzene (DNCB), CD86 expression and IL-1 β production should significantly increase, but there were no reactions in the case of SDS. However, these models were single cell culture models with few keratinocytes so they induced immunoreactions with much less immune-sensitizing compound than real human skin. In addition, it was

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difficult to apply water-insoluble samples (ex. creams or milky lotions) to these models.

Many skin models have already been established for the evaluation of water insoluble sample effects on the human skin. Bell et al.⁴⁾ have reported a full thickness human skin model consisting of the dermis and epidermis, which mimicked real human skin. Rheins et al.⁵⁾ reported a new three dimensional human skin model (Skin²™ ZK1300, constructed of fibroblasts and keratinocytes) from which, after irradiation with ultraviolet B (UVB), prostaglandin E₂ (PGE₂) and interleukin (IL)-1 α were released. Morota et al.^{6),7)} reported a new three dimensional human skin model (constructed of fibroblasts and keratinocytes) and evaluated skin irritancy against 10 compounds. The result suggested that MTT and neutral red (NR) assays should show good correlation with *in vivo* scores, but these models did not have immunoreactive cells (like dendritic cells) so they could not be used for the evaluation of skin sensitization.

The aim of this study was to construct a three-dimensional human skin model consisting of dendritic cells, keratinocytes and fibroblasts and to apply this model the *in vitro* evaluation of immune-sensitizing compounds.

2. Materials and Methods

2.1. Cells and medium

Normal human skin fibroblasts (NHSF) 46 and human epidermoid carcinoma (A431) were purchased from Riken Cell Bank (Tsukuba, Japan). Normal human dendritic cells (NHDC ; CD86+) were purchased from Cambrex Co. (USA).

2.2. Reagents

Type-I collagen was purchased from Nitta-gelatin Co. (Tokyo, Japan). Other materials were purchased from Wako Chemicals (Osaka, Japan).

2.3. Procedure of constructing three-dimensional human skin model (see Fig.-1)

Ten milliliters of 3 $\times$ DMEM, 2.2 mL of FBS, 2.8 mL of DMEM-10% fetal bovine serum (FBS), 10 mL of Type-I collagen (Cell matrix Type-I A 3.0 mg/mL, pH 3) and 5 mL of NHSF46 (2.4 $\times$ 10⁵ cells/mL) in DMEM-10% FBS were added to an ice-cooled 50-mL plastic tube and mixed, and then each 5 mL of the mixture was placed in a 60 mm culture dish with DMEM-10% FBS and cultured at 37°C, 5% CO₂, humidity above 90% for 7 days. The culture medium was changed to a mixture of DMEM-10% FBS and EpiLife KG (keratinocyte growth medium)-2 (Kurabo Co., Osaka, Japan) (1 : 1) and underwent 3-h incubation at 37°C, 5% CO₂, and humidity above 90%. After incubation, the culture medium was changed to EpiLife KG-2 and underwent 3-h incubation at 37°C, 5% CO₂, and humidity above 90%. Next, NHSF46 in collagen gel was placed on a plastic ring (outer size 2.2 cm, inner size 1.0 cm) and then placed in a six-well plate. A glass ring (Cloning ring, outer size 1.2 cm, inner size 1.0 cm, Asahi Technoglass Co., Tokyo Japan) was then placed on the collagen gel. One milliliter of Type-I collagen and 2 mL of NHDC (3 $\times$ 10⁵ cells/mL) in LGM (lymphocyte growth medium)-3-5% human plasma were added to an ice-cooled 15-mL plastic tube and mixed, and then each 0.5 mL of the mixture was placed in a glass ring for 2-h incubation at 37°C, 5% CO₂, and humidity above 90%. After incubation, A431 (1 $\times$ 10⁶ cells/mL) in 0.2 mL of EpiLife KG-2 was added to the glass ring and incubated for 1 h. Two mL of EpiLife KG-2 was then added to each well. After 24-h incubation at 37°C, 5% CO₂, and humidity above 90%, 2 mL of the mixture of DMEM-10% FBS, EpiLife KG-2 and LGM-3-5% human plasma (1 : 1 : 1) including 1.8 mmol/L of Ca²⁺ and 50 μ g/mL of ascorbic acid (three-dimensional culture medium) were added. After 24-h incubation, the medium was removed. One mL of fresh three-dimensional culture medium was added to the surface of the three-dimensional human skin model consisting of keratinocytes, dendritic cells and fibroblasts (KDF-Skin) exposed to air (Fig.-1). The three-

dimensional human skin model was incubated for 14 days to complete the structure of the skin model. In order to perform histochemical studies on the skin model, it was fixed in 10% formalin neutral buffer solution (for 24 h), embedded in paraffin, and stained with hematoxylin and eosin (HE). The surface marker expression (CD86) of NHDC was analyzed by immunostaining.

2.4. Procedure of incubating NHDC in collagen gel

One mL of Type-I collagen and 2 mL of NHDC (3×10^5 cells/mL) in LGM-3-5% human plasma were added to an ice-cooled 15-mL plastic tube and mixed, and then each 0.5 mL of the mixture was placed in a 24-well plate for 24-h incubation at 37°C, 5% CO₂, and humidity above 90%. After incubation, 0.5 mL of LGM-3-5% human plasma were added to wells, and incubated for 3 days at 37°C, 5% CO₂, and humidity above 90%. NHDC was fixed in 10% formalin neutral buffer solution, embedded in paraffin, and stained with HE. CD86 expression of NHDC was analyzed by immunostaining.

2.5. Antibodies

The primary antibody was used at 10 µg/mL for mouse monoclonal anti-human CD86 (Daco Cytomation Inc.) and the secondary antibodies were used at 0.5% for polyclonal goat anti-mouse (Daco Cytomation Inc.).

2.6. Immunostaining

HE and immunostaining were performed according to Applied Medical Research Co. The paraffin block of KDF-Skin was dried, and the paraffin removed and washed. Then 80% methanol and 0.6% hydrogen peroxide were added, and after 30-min incubation, the slides were washed with water and 3% hydrogen peroxide was added, and then after 15-min incubation, the slides were washed with water and treated with TBST (50 mmol/L Tris-HCl, pH 7.6

Fig.-1 Procedure for establishing KDF-Skin.

including 0.15 mmol/L NaCl and 0.05% Tween) for 5 min. After blocking non-specific binding with 10% normal goat serum, the primary antibody was added and incubated at 4°C overnight. Secondary antibodies were then added after washing with TBST and incubated at room temperature (r.t.) for 30 min following streptavidine reaction (r.t. for 30 min). Detection was done using 3,3'-diaminobenzidine tetrachloride (DAB) (Wako Chemicals). Slides were stained with Mayer's hematoxylin (Daco Cytomation Inc.) to identify cell morphology and negative cells in blue.

2.7. Treatment of chemicals

One mL of fresh three-dimensional culture medium was added to KDF-Skin and after 13 days' incubation, DNCB (0–2 mmol/L) in 0.2 mL of three-dimensional culture medium was put onto the surface of KDF-Skin (see Fig.-1) for 1 h. LGM-3–5% human plasma 0.5 mL was added onto NHDC in collagen gel and after 4 days' incubation, NHDC was exposed to DNCB (0–1 mmol/L) in 0.2 mL of LGM-3–5% human plasma. Culture medium was collected into microtubes after 1-h, 4-h and 24-h incubation. After 24-h incubation, 10% formalin neutral buffer solution was added to the skin model or NHDC in collagen gel, which was then stained with HE. The expression of CD86 on the dendritic cells of the skin model was analyzed by immunostaining.

2.8. LDH assay

A mixture of collected culture medium (10 µL) and PBS (40 µL) was placed into a 96-well microplate, 50 µL of reagent (nitro blue tetrazolium (0.74 mg/mL), diaphorase and NAD (50 mg/mL) dissolved in lithium DL-lactate buffer (50 mg/mL, pH 9.0)) was added to the wells and then the microplates were incubated at room temperature for 45 min. On the addition of 100 µL of 0.5 mol/L HCl as a stopping reagent, absorbance was measured at 570 nm.

2.9. IL-4 assay

Each well was rinsed immediately with Wash Buffer (0.5 mL/L of Tween 20 in PBS) and then 50 µL of cell supernatant, 50 µL of medium and 100 µL of acetylcholinesterase; IL-4 Fab' Conjugate solution was added to each well. After incubation at 4°C for 18 h, the wells were rinsed five times with the Wash Buffer, and 200 µL of Ellman's Reagent was added to the wells. Absorbance at 405 nm was then measured using a microplate reader and the concentration of IL-4 was calculated based on the absorbance of standards.

2.10. Statistical analysis

Significant differences from the control were evaluated by Student's *t*-test.

3. Results

3.1. Histochemical studies

First, we investigated the exchange of a histochemical cross-section of KDF-Skin following the incubation time (Figs.-2 and 3). After a 3-day incubation, there were two layers of epidermis (Fig.-3(a)). After a 7-day incubation, epidermis layers had increased additionally, but the horny layer was not developed (Fig.-3(b)). After an 11-day incubation, the epidermis layers were increasing, and the horny layer was observed because in the surface layer, keratinocyte nuclei disappeared and the cells became flatter in comparison with the base layer (Fig.-3(c)). After a 14-day incubation, epidermis layers were not increasing (Figs.-2 and 3(e)). The fibroblast layer did not change during 14 days' incubation (Fig.-3(d)).

3.2. Immunofunction of KDF-Skin

In order to check the immunofunction of NHDC cells in KDF-Skin, we investigated the change in the expression of CD86 on dendritic cells (cell membrane antigen of NHDC) following the incubation time by immunostaining. When incubating for 7 days, the expression of CD86+ was the strongest, but after the 14-day incubation, the ex-

Fig.-2 Effect of incubation time on the number of epidermal and horny layers in KDF-Skin.

pression of CD86+ was moderate and still observed (Fig.-4).

3.3. Effect of immune-sensitizing compounds on the immunofunction of KDF-Skin

From 0–2 mmol/L of DNCB there was no release of LDH from KDF-Skin. Exposure to 0.5 mmol/L DNCB did not significantly release IL-4, and CD86 expression did not induce the dendritic cells of the skin model ; however, 1 mmol/L DNCB induced moderate IL-4 release and CD86 expression. Two mmol/L of DNCB significantly released IL-4, and CD86 expression was strong (Table 1). On the other hand, 1 mmol/L SDS did not significantly release IL-4, and CD86 expression did not induce the dendritic cells (Table 1). One mmol/L of SDS did not significantly release LDH from KDF-Skin ; however, 5 mmol/L SDS significantly released LDH.

3.4. Effect of immune-sensitizing compounds on the immunofunction of NHDC in collagen gel

From 0–1 mmol/L of DNCB did not release any LDH from NHDC in collagen gel. Exposure to 0.2 mmol/L DNCB induced CD86 expression appearing on dendritic cells. One mmol/L of DNCB induced CD86 expression appearing on dendritic cells (Table 2). IL-4 release could not be detected.

4. Discussion

After an 11-day incubation, a horny layer was observed and after a 14-day incubation, three kinds of cells, keratinocytes, dendritic cells and fibroblasts, were alive, and the expression of CD86 in NHDC persisted. The results suggested that within 11–14 day' incubation, KDF-Skin should be used for the applied to study of effect of test chemicals on CD86 expression.

It was not clear whether the detailed mechanism of CD86 expression on dendritic cells was due to DNCB, but Aiba et al.⁸⁾ reported that 30 μ mol/L DNCB induced the CD86 expression of the monolayer of human monocyte-derived dendritic cells and anti-TNF- α or anti-IL-1 β antibodies suppressed the expression significantly, so they demonstrated that DNCB seemed to first stimulate dendritic cells to secrete IL-1 β or TNF- α which then induced their CD86 expression. Staquet et al.⁹⁾ reported that 38 μ mol/L DNCB induced the CD86 expression of the monolayer of dendritic cells generated from human monocytes. Yoshida et al.³⁾ reported that 3 μ g/mL DNCB released IL-1 β and induced CD86 expression in THP-1 cells. Coquette et al.¹⁰⁾ reported that 1 mg/mL DNCB released IL-1 α and IL-8 from reconstructed human epidermis. Ryu et al.¹¹⁾ reported that IL-4 and granulocyte-macrophage colony-stimulating factor increased the expression of CD86. Immune-sensitizing compounds such as DNCB, methylparaben, and benzethonium chloride also released IL-1 β and IL-4 from the A431 monolayer.¹²⁾ These results suggested that DNCB should release cytokines (IL-1, IL-4, TNF- α , etc.) from A431 and induce CD86 expression in NHDC. Further study should investigate the effect of DNCB on cytokines and the effect of anti-cytokine antibody on CD86

Fig.-3 Histological cross-section of KDF-Skin after staining with hematoxylin and eosin. Skin model was incubated for 3 days (a), 7 days (b), 11 days ((c) and (d)) or 14 days (e).

Fig.-4 Histological cross-section of KDF-Skin immunostaining with CD86. Skin model was incubated for 3-14 days. CD86 immunostaining : -, blue ; ±, gray ; +, light brown ; + to ++, brown ; ++, dark brown.

Table-1 Effect of DNCB and SDS concentration on IL-4 release from KDF-Skin.

Compound	CD86 expression	IL-4 release (pg/mL) ^a
Control	+	26.8 ± 10.4
DNCB 0.5 mmol/L	+	30.1 ± 10.4
DNCB 1 mmol/L	+ to ++	39.3 ± 17.6
DNCB 2 mmol/L	++	61.2 ± 20.4*
SDS 1 mmol/L	+	29.5 ± 11.8

^a Data are the means ± S.D. of three or four values.

CD86 immunostaining : +, light brown ; + to ++, brown ; ++, dark brown.

IL-4 release measured after 24-h incubation.

Significantly different from the control group, * $p < 0.05$.

Table-2 Effect of DNCB concentration on CD86 expression of NHDC in collagen gel.

DNCB concentration (mmol/L)	CD86 expression
0	+
0.2	+ to ++
1	++

CD86 immunostaining : +, light brown ; + to ++, brown ; ++, dark brown.

CD86 expression measured after 24-h incubation.

expression in NHDC. Less DNCB induced CD86 expression in the dendritic cell monolayer or the dendritic cell in collagen gel on comparison with CD86 expression in KDF-Skin. These results suggested that because of the barrier-effect of keratinocytes, a higher amount of DNCB induced CD86 expression in KDF-Skin than in a human skin model consisting of only dendritic cells, and that the skin model might overestimate the immunoreaction of real skin in comparison with KDF-Skin. In addition, our skin model has the advantages of

- 1) The application of water-insoluble samples (organic compounds and cosmetics, etc.).
- 2) A long life for culturing dendritic cells.
- 3) The ability to investigate the indirect effect of test chemicals on dendritic cells, like CD86 expression.

On the other hand, our skin model has the following disadvantages :

- 1) The horny layer of keratinocytes in this skin model is thinner than that of keratinocytes in real human skin.
- 2) Evaluation of CD86 expression in the dendritic cell is qualitative.

Further study should be done to solve these problems.

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ケラチノサイト、樹状細胞、線維芽細胞を含む3次元培養ヒト皮膚モデルの 構築と皮膚感作性の動物実験代替法への応用*

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皮膚感作性のインビトロ評価法を開発するために、われわれはケラチノサイト、樹状細胞、線維芽細胞を含む3次元培養ヒト皮膚モデルの構築を試みた。皮膚モデル中の細胞の生死はHE染色を行って観察した。角層形成後の11～14日目のモデルを試験に用いた。細胞毒性を発現しない濃度のDNCB添加時において、ケラチノサイト、樹状細胞からのIL-4放出および樹状細胞のCD86発現が誘導された。一方、非感作性物質のSDS添加時はIL-4放出およびCD86発現の誘導は見られなかった。これらの結果から、この皮膚モデルが皮膚感作性物質の動物実験代替法に適用できる可能性が示唆された。