Cell Chip-Based Monitoring of Toxic Effects of Cosmetic Compounds on Skin Fibroblast Cells

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The present study estimated the efficacy of electrochemical detection of imidazolidinyl urea-induced cell toxicity in skin human fibroblast cells (HFF cells). The gold nanopunct structures were fabricated through a nanoporous alumina mask, and the structural formations were confirmed via scanning electron microscopy. The HFF cells were allowed to attach to RGD (Arg-Gly-Asp) peptide nanopatterned surfaces, and electrochemical tools were applied to skin cells attached to the chip surface. The HFF cells evidenced inflammation responses to allergens such as imidazolidinyl urea. The cells were subsequently treated with different concentrations of imidazolidinyl urea for 24 h in culture, which induced a change in the cyclic voltammetry (CV) current peak. Treatment with imidazolidinyl urea induced a loss of cell viability and accelerated inflammation in a concentration-dependent manner. The expression level of inflammation-related proteins such as IL-1 beta were increased in imidazolidinyl urea-treated cells. The CV results demonstrated that imidazolidinyl urea significantly reduced the current peaks in a dose-dependent manner. The results showed that the current peak was reduced in accordance with the increases in imidazolidinyl urea-induced inflammation. In conclusion, the results of this study suggest that the electrochemical-based chip provides crucial information for improvements to a cell chip system for drug screening applications.

Keywords: Imidazolidinyl Urea, Cyclic Voltammetry, Skin Fibroblast Cell, Toxicity.

1. INTRODUCTION

Preservatives are an important class of chemicals used to inhibit the growth of microorganisms harmful to industrial products.¹ Under in vivo conditions, they are toxic to microorganisms as well as humans.² In particular, antimicrobial preservatives such as imidazolidinyl urea (IU) are used widely in the formation of pharmaceutical creams and ointments, as well as in cosmetics and toiletries.³ It is known to cause allergic contact dermatitis in patients who use these products.⁴ Additionally, IU is known as a formaldehyde-releasing agent, and it may carry out its activities by releasing formaldehyde or via the action of the parent chemical structure of the compounds.⁵

Generally, predictive testing for the capacity to induce allergic contact dermatitis is part of the safety assessment of new ingredients used in cosmetics and topically applied drugs.⁶ Thus far, the identification and evaluation of unknown sensitizers relies on animal testing, mainly the guinea pig maximization test, the Bühl test, and local lymph node assay (LLNA), as no validated alternative currently exists.³⁷ The additional testing of chemicals for their allergenic potential as required by the new EU-legislation on chemicals is anticipated to significantly increase the use of animals.⁸ It has been previously estimated that skin sensitization testing is among those effects that would require large numbers of animals.⁵⁹ Conversely, the 7th amendment to the cosmetics directive completely bans all animal testing for cosmetic ingredients for all human-health related effects by 2009 for all endpoints, with the exception of the repeated-dose toxicity endpoints-for which the marketing ban deadline is 2013.³⁴ Therefore, in the screening of drugs, cosmetics and other chemicals for human use, it is very important, both from the safety and economic perspectives, to identify biological markers to discriminate allergy and irritation, events which have different impacts on human health.³⁸ The development of methods for the prediction of skin
sensitization potential without animal testing has been an active research area for some time, but has received further impetus in the current environment of existing and pending EU regulations.3,4 The ultimate objective is the establishment of in vitro assays ready for validation. Additionally, the need for sensitive, short-term tests for aquatic hazard assessment and biomonitoring has led to an increasing use of gametes in applied toxicology.

To the best of our knowledge, there have been no reports regarding the effects of the preservatives tested herein. Therefore, we attempted to determine whether these compounds are capable of inducing toxicity under in vitro conditions. The effect on cell viability was monitored by the 3-[4,5 dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) test and cyclic voltammetry analysis (CV). Additionally, we designed a highly sensitive cell chip that can be used to monitor the effects of imidazolidinyl urea on skin fibroblast cells based on the electrochemical method on nanogold plates, including at picomolar concentrations.

2. EXPERIMENTAL DETAILS

Preparation of Gold nanopunct Well-ordered nanoporous alumina masks were prepared from aluminum foil (99.99%, 100 um in thickness) via a two-step anodization process.11 Aluminum foil was electro-polished in a solution of HClO4 and ethanol (1:4, v/v) at a constant voltage of 20 V for 60 s. The first anodization step was conducted via the application of a DC voltage of 40 V in 0.3 M oxalic acid solution at 3 °C for 4 h. A Teflon sample holder with a circle hole with a 10 mm diameter was employed. The alumina layer formed during the first anodization process was dissolved in a mixture solution of 0.4 M phosphoric acid and 0.2 M chromic acid for 5 h at 65 °C. After removing the alumina layer, a second anodization process was carried out for 5 min under the same conditions employed for the first anodization. After the second anodization, the alumina layer was etched slightly by 15 min of immersion in aqueous 5 wt% phosphoric acid at 30 °C. Then, the surface of the nanoporous layer was painted with a protective layer consisting of a mixture of nitrocellulose and polyester resin in butyl acetate, ethyl acetate, and isopropyl alcohol. The remaining aluminum substrate was removed in saturated HgCl2 solution. The alumina layer was again etched for 8 min in aqueous 5 wt% phosphoric acid. Finally, the protective layer was dissolved in acetone and rinsed several times in distilled water, then sorted on filter paper. The ITO glass substrate was placed on the alumina mask and turned upside down. The filter paper was then carefully peeled from the alumina mask.

2.1. Fabrication of a Cell-Based Chip

The alumina mask with through-holes on the ITO glass was placed on a sample holder in an evaporator system. The gold was deposited on the ITO substrate through the pores of the nanoporous alumina mask using a thermal evaporator (ULVACVPC-260) with a vacuum pressure of 3 × 10−6 Torr and an evaporation rate of approximately 0.1 Å s−1. After gold deposition, the alumina mask placed on the ITO substrate was dissolved for 5 minutes in 10 wt% NaOH and then rinsed 3 times in distilled water. After the pretreatment, a well-ordered oligopeptide (RGD-MAP-C) was fabricated on the freshly cleaned gold electrode as reported in our previous work. For the electrochemical measurements, a 1 cm × 1 cm × 0.5 cm (width × length × height) cell chip chamber was fabricated via the fixing of a plastic chamber (Lab-Tek(R), Thermo Fisher Scientific, Waltham, MA). Afterward, skin fibroblast cells were seeded on the chip at a density of 5 × 10^4 cells/chip and then immobilized via RGD integrin interaction for 24 h in a standard cell culture environment. The surface morphologies were analyzed using a scanning electron microscope (SEM) (ISI DS-130C, Akashi Co., Tokyo, Japan).

2.2. Cyclic Voltametry Analysis

The Cyclic Voltametry analysis was conducted using a potentiostat (CHI-660, CH Instruments, Austin, TX, USA), controlled with “General Purpose Electrochemical System” software. The homemade three-electrode system was composed of a cell-based chip working electrode, a platinum wire auxiliary electrode and an Ag/AgCl reference electrode. The effect of anticancer drugs on cell behavior, as well as the electrical properties of living cells, was investigated with the electrode system. PBS (10 mM, pH 7.4) was employed as an electrolyte at a scan rate of 0.1 V/s.

2.3. Cytotoxicity Analysis

The cell viability was assessed via MTT analysis. The cells (5 × 10^4 cell/ml) were treated with IU at various doses. Following 24 h of incubation, an MTT solution was added and the cells were incubated for 4 h at 37 °C. The supernatant was then removed, and 200 μl of dimethyl sulfoxide was added. The percentage of viable cells was determined by measuring the absorbance at 540 nm using a microplate reader.

2.4. Assay for IL-Beta

Cultured cells (98-mm diameter dishes) were stimulated with IU for 24 h at various concentrations in DMEM. The conditioned medium was collected at the end of the incubation, and IL-1 beta concentration was measured using an ELISA kit. The absorbance of each sample at 450 nm and 540 nm was measured with an ELISA reader (Bio-Tek, USA). Absorbance was corrected with reference to a standard curve.
3. RESULTS AND DISCUSSION

3.1. SEM Image of Gold Nanopunct Array

The two-step chemical wet etching process for production of the alumina with through-holes is shown in Figure 1(a).

Al mask  
Gold nanopunct substrate

Fig. 1. SEM image of alumina mask and gold nanopunct arrays fabricated on an ITO glass substrate.

The average diameter of the nanoporous alumina was measured at 70 ± 5 nm via SEM analysis. The thickness of the alumina mask was approximately 200 nm. The thickness of the nanoporous alumina mask depended on the second anodization time. Thus, the long-range ordered ultra-thin alumina mask with through-holes was placed on the ITO glass substrate. It can bind to the substrate via van der Waals interactions. Figure 1(b) shows an SEM image of the gold nanopunct arrays formed on ITO glass after the removal of the nanoporous alumina mask. The average diameter of gold nanopunct arrays was approximately 70 ± 5 nm, whereas the mean height was 45 ± 5 nm. The shape of the gold nanopunct arrays depends on the pore diameter and thickness of the alumina mask. Generally, the nanoporous alumina mask played a very important role in the ordering of the nanopunct array, and was removed after the second etching process. Thus, the gold nanopunct with a regular size and uniform spatial distribution was achieved using the nanoporous alumina mask, for use as a nanobioplatform for the cell chip.

3.2. Cell Image in the Two-Dimensional Gold Nanopunct Array

The self-assembly was used to produce a thin biomolecular layer on a solid surface for various electronic device applications. The gold surface has been used as a substrate for the formation of self-assembled monolayers of thiocompounds such as cysteine (Cys) proteins. In particular, the cell adhesion motif RGD (Arg-Gly-Asp) and its derivatives, which are found in a variety of cellular adhesive molecules, have been widely employed as substrates to increase cellular attachment, adhesion, and proliferation. In particular, a modified RGD peptide terminated with a Cys protein can self-assemble on Au sensing surfaces. Focal contacts function as coordinate sites between the extracellular matrix (ECM) and the actin cytoskeleton. The size of the focal contact is correlated positively with the attachment strength and inversely correlated with the migratory rate. In this study, skin fibroblast cells were applied to gold nanopunct arrays modified with a RGD peptide layer. The morphology of the cell was investigated via inverted optical microscopy (Fig. 2). The cell images showed control skin fibroblast cells and IU-treated cells. The skin fibroblast cells on the RGD Au surface appeared well-spread and evidenced round or elongated morphologies. However, IU-treated cells resulted in a reduction in the cell number and complete lysis at 100 μM doses. The significant changes in cell morphology after incubation with IU provided further evidence that the IU retained its toxicity in the skin fibroblast cells on the self-assembled gold nanopunct array cell chip.

The cytotoxicity of IU was determined via the MTT assay (Fig. 3(a)). The cell viability of the IU was evaluated at concentrations of 10, 50, 100, 500, and 1000 μM, respectively. IU was not cytotoxic at a 10 μM dose concentration against the skin fibroblast cells. However, the cell viability was reduced from 76.86% to 13.03% as the IU concentration was increased from 50 to 1000 μM. Amouox et al. have reported acute cytotoxicity of IU in sea urchin eggs, primarily as the result of the inhibition of protein and DNA synthesis. In previous reports, the cell toxicity of leukemia cells was dose-dependent, and apoptosis and caspase-3 activity was shown to occur at a 0.1% concentration. Our results demonstrated that the cell viability of IU occurred in a dose-dependent fashion. The release of IL-1 beta was measured in supernatants 24 h after treatment by ELISA. As shown in Figure 3(b), a dose-related increase in IL-1 beta release was noted following exposure to all contact allergens tested. At present, the identification of potential contact allergens relies wholly on animal testing. It is, therefore, a matter of great urgency to develop reliable in vitro alternative methods to replace animal testing. Among the recently proposed tests, the release of IL-1 beta is one of the most promising biomarkers used to distinguish sensitizers from non-sensitizers. In skin fibroblast cells, the level of IL-1 beta increases in a dose-dependent manner.

Electrochemical study of IU and cyclic voltammetry analysis of cell immobilized electrode. IU, a urea derivative, functions as a formaldehyde donor. The formaldehyde
reacts most rapidly with organic and inorganic anions, amino and sulphide groups, and electron-rich groups to disrupt the metabolic process, ultimately causing the death of the organism. There is a growing amount of evidence to suggest that several methylated compounds are potential formaldehyde generators, the biological reactions of which can induce apoptosis. We conducted cyclic voltammetry analyses of IU from 10 uM using a potential window of −0.2 V to 0.8 V (vs. Ag/AgCl) and a scan rate of 100 mV s⁻¹. IU exhibited a cathodic ($E_{pc}$) peak current at 19 mV and an anodic ($E_{pa}$) peak current at 212 mV at a 10 uM IU concentration (Fig. 4). The voltammetric behavior of IU may be associated with the presence of amino and sulphide groups and electron-rich groups.

Skin fibroblast cells were seeded and incubated on a gold nanopunct array at a density of 5 × 10⁴ cells/ml for 24 h. Afterward, the cells were exposed to different doses of IU (10 nM–100 pM) dissolved in the culture medium for 24 h in order to determine the effects of IU on the cell viability and allergic contact dermatitis. The cells on the electrode were rinsed carefully with PBS and subjected to electrochemical measurements. The electrolyte used was a solution of 0.01 M PBS (pH 7.4). The electrochemical potential of the skin fibroblast cells on a chip was measured at a constant potential of 100 mV s⁻¹ versus Ag/AgCl. CV signals from skin fibroblast cells at various doses of IU ranging from 10 nM to 1 nM evidenced a dose-dependent relationship, corresponding to the reduction in peak current, $I_{pa}$ value (Fig. 5(a)). Since IU at 100 pM concentrations stimulated cell proliferation, the peak current, $I_{pa}$, might be increased by an increase in cell number. Additionally, Figure 5(b) shows the representative CV of redox phenomena from skin fibroblast cells immobilized on a peptide-fabricated Au electrode at IU concentration (100 pM). A pair of well-defined redox peaks from skin fibroblast cells was observed at 25 mV and 176 mV (vs. Ag/AgCl) as anodic ($E_{pa}$) and cathodic ($E_{pc}$) peak potentials, respectively. The peak separation $|E_{pc} - E_{pa}|$ between the anodic and the cathodic peaks was approximately 151 mV, and the peak current ratio $I_{pa}/I_{pc}$ was greater than 1, indicating the distinct linear characteristics of the cell. No redox peaks were observed from the 12 h electrodes on which no cells were immobilized (Fig. 5(b)). The effects of IU on the detection of electron transfer from immobilized cells to an electrode were assessed to find that the peptide-modified Au electrode yielded more stable and stronger redox peaks on the CV than was observed with a bare Au electrode. This
result was related closely to cell adhesion strength on a working electrode. Hence, after cell immobilization, the peaks on the CV could be regarded as the signals produced by the redox behavior of skin fibroblast cells. Remarkably, the $I_{pa}$ peaks at 24 h after IU treatment evidenced significant signal differences for the cells treated for different times on the electrode surfaces. At 36 h, the current peak increased with the increasing times of drug treatment of cells. But, the anodic oxidation peak was not showed from the 36 h electrodes (Fig. 5(b)). Thus, our results show that the optimal detection time of IU occurred at 24 h. Additionally, a 100 pM concentration of IU on the skin fibroblast cells resulted in distinct CV peaks. In our study, at a 10 nM concentration of IU using the MTT assay evidenced no toxicity. However, the CV peaks of IU were detected at a 100 pM concentration. Thus, the CV assay data proved more sensitive and resulted in stronger toxicity peaks than MTT analysis.

4. CONCLUSIONS

A cell-based chip was fabricated by immobilizing living cells on RGD-MAC-C peptide self-assembled gold electrodes to evaluate the in vitro effects of IU. Using this cell line, we have demonstrated that the exposure of skin fibroblast cells to allergens results in a dose-related release of IL-1 beta. The detection range of a fabricated cell chip was 0–10 nM, and a specific voltammetric peak was observed at a 100 pM concentration of IU in the skin fibroblast cells. The optimal detection time of IU evidenced a CV peak at 24 h. These results indicate that electrochemical signals from cells treated with IU evidenced more sensitivity and more profound range than MTT analysis. The results demonstrated that the current peak was reduced according to increases in imidazolidinyl urea-induced inflammation. Additionally, these data demonstrate the utility and efficacy of monitoring this IU-specific effect for assessments of contact sensitization risk in a human in vitro assay.

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References and Notes


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