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Evaluation of the effects of chitin nanofibrils on skin function using skin models

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ABSTRACT

Chitins are highly crystalline structures that are predominantly found in crustacean shells. Alpha-chitin is composed of microfibers, which are made up of nanofibrils that are 2–5 nm in diameter and 30 nm in length and embedded in a protein matrix. Crystalline nanofibrils can also be prepared by acid treatment. We verified the effect of chitin nanofibrils (NF) and nanocrystals (NC) on skin using a three-dimensional skin culture model and Franz cells. The application of NF and NC to skin improved the epithelial granular layer and increased granular density. Furthermore, NF and NC application to the skin resulted in a lower production of TGF-β compared to that of the control group. NF and NC might have protective effects to skin. Therefore, their potential use as components of skin-protective formulations merits consideration.

1. Introduction

Alpha-chitin is composed of microfibers, which are made up of nanofibrils (NF) that are approximately 2–5 nm in diameter and 30 nm in length and embedded in a protein matrix (Chen, Lin, McKittrick, & Meyers, 2008; Fabritius et al., 2012; Nikolov et al., 2011; Raabe et al., 2006). Isolated chitin NF show a potential for use in drug delivery systems, the tissue engineering of scaffolds, and wound dressing (Muzzarelli et al., 2007). Acid hydrolysis is one of the main methods used to prepare chitin NF (Gopalan & Dufresne, 2003; Revol & Marchessault, 1993). Moreover, ultrasonication of squid pen beta-chitin under acidic conditions yields 3–4-nm-wide chitin NF with relatively low crystallinity (Fan, Saito, & Isogai, 2008). Chitin nanofibers are easily prepared using either fine grinding or wet-type atomization methods (Ifuku & Saimoto, 2012).

Chitin and chitosan have an accelerating effect on the wound healing process and regulate immune response (Muzzarelli, 2009, 2010). Recently, Chitin and chitosan could be used as nanofibrils. However, it is unclear whether chitin NF could maintain original function or not.

We have previously found that chitin NF improve the clinical symptoms and suppress the onset of ulcerative colitis in an animal model (Azuma et al., 2012). Furthermore, chitin NF suppressed myeloperoxidase activation in the colon and decreased serum interleukin (IL)-6 concentrations. In contrast, the application of chitin powder did not produce any anti-inflammatory effect (Azuma et al., 2012).

Because many people exhibit skin hypersensitivity in response to cosmetics and textiles, the development of materials exempt from inflammatory activity is essential. In the present study, we describe a novel three-dimensional skin model assay to evaluate the skin-protective effects of chitin NF.

2. Materials and methods

2.1. Preparation of chitin nanofibers and chitin nanocrystals

Chitin nanofibers and nanocrystals were prepared as described previously (Gopalan & Dufresne, 2003; Ifuku et al., 2010). In brief,

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dry chitin powder from crab shell was dispersed in water at 1 wt.%, and acetic acid was added to adjust the pH value to 3 to facilitate fibrillation. The chitin was roughly crushed with a domestic blender. Finally, the slurry was passed through a grinder (MKCA6–3; Masuko Sangyo Co., Ltd.) at 1500 rpm. Chitin crystals were prepared by hydrolyzing the chitin with 3 N HCl at the boil for 90 min under stirring. After acid hydrolysis, the suspension was washed with distilled water by centrifugation thoroughly. The precipitate was dispersed in water at 1 wt.%, and acetic acid was added to adjust the pH value to 3. The chitin was passed through a grinder.

2.2. Test materials

Test using 3D human skin culture model: we prepared a 1% chitin NF dispersion and a 1% chitin nanocrystal (NC) dispersion at pH 3 and pH 6 by the grinding method described by Ifuku and Saimoto (2012). Control solutions were distilled water, or acetic acid at pH 3 and pH 6 (AC); 1% N-acetyl-D-glucosamine (GlcNAc) was used as a positive control for effect of the keratinocyte growth factor (Minami & Okamoto, 2010).

Test using Franz diffusion cells: we prepared samples of 1% chitin nanofibrils and nanocrystals under the following conditions (Ifuku & Saimoto, 2012): pH 6, pH 6 Ac, and DW.

2.3. Test procedures using 3D human skin culture model

The three-dimensional epidermal model LabCyte EPI-MODEL 24 (Tissue Engineering Japan Co., Aichi) was used to test the contact effect of each sample. This model, which includes skin layers between the keratin layer and the basal layer, was incubated at 37 °C in a 5% CO_2 humidified atmosphere.

The 9 conditions tested in this experiment are as follows: (1) no application (NON) ($n = 8$); (2) distilled water (DW) ($n = 12$); (3) 1% acetic acid (AC) solution, pH 3 ($n = 8$); (4) 1% AC solution, pH 6 ($n = 8$); (5) 1% chitin NF dispersed at pH 3 ($n = 8$); (6) 1% chitin NF dispersed at pH 6 ($n = 12$); (7) 1% chitin NC dispersed at pH 3 ($n = 8$); (8) 1% chitin NC dispersed at pH 6 ($n = 12$); and (9) 1% GlcNAc ($n = 12$).

The assay media (0.5 mL/well) (Tissue Engineering Japan Co., Aichi) was aliquoted into the assay plate (Tissue Engineering Japan Co., Aichi). Next, the three-dimensional skin culture model was placed in each well. The assay plates were then incubated in a CO_2 incubator for 1 h. The test material (50 μL/well) was applied to the keratin layer of the three-dimensional skin culture model after the 1-h incubation.

The three-dimensional human skin culture models were removed from the assay plate at 4, 12, and 24 h post-application, and the cultured skin (from the keratin to basal layers) were harvested.

2.4. Histological observations

The harvested skin culture was immersed and fixed in 10% formalin (Mildform 10 N, Wako Pure Chemical Industries Ltd., Osaka, Japan). Cross-sectional slices (4-μm-thick) were soaked in hematoxylin and eosin (H&E) and histological evaluations were conducted. The samples were prepared by Sumika Technoservice Corporation (Osaka, Japan). The H&E stained samples from each experimental group were evaluated using an optical microscope (BX51-FL, Olympus Corporation, Tokyo). The tissue image analysis was done using Lumina Vision (Ver. 2.5.2.1, Mitani Corporation, Tokyo, Japan).

Images for 4 non-continuous fields of view at 400× magnification were taken for each H&E-stained sample in each experimental group. Sample scores were calculated by averaging the observational scores from one quadrant of the image for the granular layer and stratum spinosum. The below criteria were used to convert the observations into a score.

2.5. Assessment of the granular layer and the stratum spinosum

The number of granular layers and the granule cell density of the layers were evaluated.

A complete absence of the granular layer was assigned a score of 0; a partial granular layer was assigned a score of 1; if 1, 2, 3, or 4 layers were present, scores of 2, 3, 4, and 5, respectively, were assigned.

The granule cells lacking keratohyalin granules were assigned a score of 0, a weak presence was assigned a score of 1, a moderate abundance with a high dye-affinity was assigned a score of 2, and a granule cell with abundant granules was assigned a score of 3.

The stratum spinosum quality was evaluated by measuring the width of the intercellular gap and the clarity of the nucleus. A score of 0 was assigned if the intercellular contacts in the stratum spinosum had collapsed; (1) if the intercellular gap was greater than 1 mm at 400× magnification; (2) if the space was less than 1 mm; and (3) if a space could not be confirmed by the naked eye, when I displayed the image which photographed at magnification 400 times with 122 mm in height, size of 163 mm in width In addition, a score of 1 was assigned if the nucleus was clearly visible; otherwise, a score of 2 was assigned.

2.6. Test procedures using Franz diffusion cells

Franz diffusion cells (PermeGear Inc., Keystone Scientific K.K., Japan) were used to evaluate the test model. Skin removed from Hos:HR-1 mice (8–9 week-old, 25–35-g males, Hoshino Laboratory Animals, Inc., Ibaraki, Japan) was used. The use of the animals and the procedures they underwent were approved by the Animal Research Committee of Tottori University.

The 4 experimental conditions were as follows: (1) DW ($n = 4$); (2) 1% AC solutions of pH 6 ($n = 4$); (3) 1% chitin NF dispersed at pH 6 ($n = 4$); and (4) 1% chitin NC dispersed at pH 6 ($n = 4$).

Skin samples removed from mice sacrificed by cervical dislocation were applied to 1× phosphate buffered saline (PBS 10×, pH 7.4; Life Technologies Corporation, Tokyo, Japan)-soaked Franz cells. The cells were incubated in a thermostat chamber for 1 h (TERMO MINDER EX; Taitec Corporation, Saitama, Japan). Each test material was then applied and the cells were re-incubated. A graphical illustration of the methods is shown in Fig. 1.

At 1, 3, and 6 h post-application, PBS was aspirated from the cells. Approximately 1 and 3 h after removal of PBS, fresh PBS was injected into Franz cells using a disposable feeding needle (FG6206; Fuchigami, Kyoto, Japan), and reincubated. The aspirated PBS was concentrated using a centrifuge (SCT 5BA, Hitachi Koki Co., Ltd., Tokyo) at 4000 × g for 20 min at room temperature. The concentrated PBS was stored at −80 °C until analysis.

2.7. Measurement of cytokine concentrations in PBS

We measured the concentration of IL-1α and TGF-β using a commercially available cytokine measurement kit (Mouse, ELISA Kit, Quantikine M (96 well), R&D Systems, Minneapolis, USA). In addition, we calculated the cumulative cytokine production level through the summation of cytokine concentrations at each time point.

2.8. Statistical analysis

Analysis was performed using 4 step Excel Statistics (OMS Publishing, Saitama, Japan). For each investigation, we performed the