The Influence of Glycation in Three-Dimensional Human Cultured Epidermis

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Abstract

Destination: The influence by the addition of glucose and glyoxal was studied in three-dimensional human cultured epidermis. Methods: The glucose concentration (3-5g/L) of the medium for a three-dimensional human cultured epidermis (MEL-300) was changed and AGEs intermediate, glyoxal (GO)(0.3 μg/mL) were added, and it was cultivated at 37°C for 14 days. The amount of protein, Nε-(carboxymethyl) lysine (CML), pentosidine and fluorescence intensity from AGEs of the tissue homogenate was measured. In addition, the tissue was observed by hematoxylin & eosin (HE) staining and by the immunostaining method using anti-CML antibody. Results: Epidermis thinning was histogenetically observed by the addition GO. CML was identified at the horny cell layer by the immunostaining technique. The amount of CML was increased by the addition of GO to three-dimensional human cultured epidermis. Pentosidine was not detected. When it was subjected to excitation by a light-emitting diode (LED) of wavelengths of 365-370 nm (lamp A) and 370-375 nm (lamp B), the detected fluorescence wavelength had peaks of 423 nm, 456 nm and 541-573 nm in using lamp A, 421 nm and 543-585 nm in using lamp B and was increased by the addition of glucose and GO. Conclusion: The experiment system using three-dimensional human cultured epidermis could be used as a model of glycation in skin by going by the amount of CML and fluorescence intensity from AGEs.

KEY WORDS: skin, advanced glycation end products (AGEs), Nε-(carboxymethyl) lysine (CML), pentosidine, glyoxal
three days and it was cultivated for two weeks. Cultured epidermis. The culture medium was exchanged every control group were added to the three-dimensional human control group and 100 μL of distilled water in the negative μg/mL) corresponding to each experiment using 40% solution was added. 100 μL of GO regulated concentration (0.0, 0.3, 3 6 well plate and 5mL of the new warmed maintenance medium Washers were placed and then stacked in pairs in the center in a 120mL of maintenance medium was regulated in the pentosidine and the sample for determination of protein concentration were prepared. Each three-dimensional human cultured epidermis for 2 weeks was placed in an Eppendorf tube/disk and 1mL of 50mM Tris HCl (pH 8.0) 1% SDS prepared 2-amino-2-hydroxymethyl -1,3- propanediol (Tris; Wako Pure Chemical Industries), acium hydrochloricum, sodium dodecyl sulfate (SDS; Wako Pure Chemical Industries) was added and the samples were homogenated with storage ice by hand type homogenizer (Funakoshi, Bunkyo-ku, Tokyo, Japan) for 3 minutes. After they were centrifuged at 10,000 rpm for 15 minutes, the supernatant was collected in another Eppendorf tube and 100 μL of 14% PCA prepared 70% perchloric acid (PCA; Wako Pure Chemical Industries) was put in. After still standing cooling using ice for 20 minutes and centrifuging at 10,000 rpm for 15 minutes, the supernatant was put in 1 mL of Tris HCl (50 mM, pH 8.0). The screening experiment was performed in a manner to homogenate the cultured cell, and we confirmed that the sensitivity of detection of CML decreased by protease treatment, so we decided that protease treatment would not be performed.

CML Measurement
CML was analyzed with the ELISA test using CML /N -(carboxymethyl) lysine ELISA Kit (CircuLex, Ina, Nagano, Japan). The samples were reacted using anti-CML monoclonal antibody as the first antibody and HRP labeled anti-mouse IgG antibody as the second antibody, and the samples were measured at 450 nm (dominant wavelength)/540 nm (reference wavelength) using an ARVO MX 1420 Multilabel Counter microplate reader (Perkinelmer Japan, Yokohama, Kagawa, Japan). The amount of protein was measured using a DC Protein Assay Kit (Bio-Rad, Shinagawa-ku, Tokyo, Japan). The standard curve was prepared using human serum albumin (HSA; Sigma-Aldrich, Shinagawa-ku, Tokyo, Japan) prepared to 1 mg/mL. 40 μl of each sample was added to each well, 20 μl of reagent A was added, and then 160 μl of reagent B was added. It was agitated at room temperature for 30 minutes and the absorbance at 750 nm was measured by a microplate reader (ARVO MX 1420 Multilabel Counter). CML per protein in tissue was computed from the result.

Histofluorescence measurement
The fluorescence value of the three-dimensional human cultured epidermis was measured from an upper surface using the fluorescence value of skin measuring machine produced experimentally in our laboratory. Two kinds of light-emitting diodes (LED) (NITRODE SEMICONDUCTORS. Co., Ltd. Naruto, Tokushima, Japan) which had the wavelengths of 365-370 nm (lamp A) and 370-375 nm (lamp B) as excitation light were used and a C10988MA type mini spectral apparatus (Hamamatsu Photonics, Hamamatsu, Shizuoka, Japan) was used in fluorescence analysis.

Statistical significance test
Statistical significance test was analyzed by multiple comparison of Tukey.
**Results**

**Tissular investigation**

An HE stained image of the cultured tissue is shown in *Fig.1*. When the glucose concentration of the medium was raised from 3.0 g/L to 5.0 g/L, it was likely to broaden the gap of epidermal tissue. When the GO concentration in the medium was raised from 0 to 0.3 μg/mL, it was likely to thicken the gap of epidermal tissue.

**Amount of CML in tissue**

The amount of CML in cultured tissue is shown in *Fig.2*. When the glucose concentration of the medium was 3.0 g/L, the amount of CML was significantly increased from 0.01 ± 0.003 μg/μg protein to 0.095 ± 0.021 μg/μg protein by raising the GO concentration from 0 to 0.3 μg/mL (*p*<0.001). When the glucose concentration of the medium was 5.0 g/L, the amount of CML was significantly increased from 0.012 ± 0.03 μg/μg protein to 0.075 ± 0.012 μg/μg protein (*p*<0.001).

**Amount of pentosidine in tissue**

Under the condition that the glucose concentration of the medium was 3.0-5.0g/L and the GO concentration was 0-0.3 μg/mL, the amount of pentosidine in tissue below measurable limits (<0.0004 μg/μg).

**Immunohistochemical investigation of CML**

An immunostaining image of the cultured tissue is shown in *Fig.3*. Under the condition that the glucose concentration of the medium was 3.0g/L and the GO concentration was 0 μg/mL, CML was immunohistochemically unaccounted for. CML was stained by raising the glucose concentration of the medium from 3.0g/L to 5.0g/L. CML was strongly stained in the horny cell layer by adding GO to the medium.

![Fig. 1. Histology of 3-dimensional skin model. Tissue is stained by hematoxylin and eosin.](image1)

![Fig. 2. The change of CML amount by changing the glucose and GO concentration in the medium. Data are expressed as mean ± standard deviation and analyzed by Tukey test (n=5). CML, carboxymethyl-lysine; GO, glyoxal; Glc, glucose.](image2)
**AGEs fluorescence of skin cultured tissue**

The measured result of AGEs fluorescence intensity by fluorescence detector under the condition that the glucose concentration of the medium was 3.0-5.0 g/L and the GO concentration was 0-0.3 μg/mL is shown in Fig. 4, 5.

When lamp A was used, the peak tops at the wavelengths of 423 nm, 456 nm, and 541-573 nm were accounted for. The fluorescence intensity was increased by raising the GO concentration.

When lamp B was used, the peak tops at the wavelengths of 421 nm and 543-585 nm were accounted for. The fluorescence intensity was increased by raising the glucose and GO concentration.

**Fig. 3.** Immunohistochemistry of 3-dimensional skin model using anti-CML antibody. CML; carboxymethyl-lysine, GO; glyoxal, Glc; glucose.

**Fig. 4.** Fluorescence intensity value in 3-dimensional skin model using lamp A. Glc; glucose, GO; glyoxal.
Discussion

In this study, we investigated the effect from putting glycation stress on the medium of a three-dimensional human cultured epidermis for 2 weeks, and the accumulation of CML and other fluorescent AGEs and tissue damage were showed.

The load of glycation stress was adding glucose and GO to the medium in this experiment. GO is one of the intermediates of glycation reaction, and CML, which is the most notable AGEs accumulating in the skin, is formed from GO. CML is reported to induce apoptosis by adding collagen arising from CML to human fibroblast 6). In the result, it is guessed that collagen production deterioration in skin and CML is responsible for making skin less resilient.

It was confirmed that the amount of CML in tissue was increased by the load of glycation stress. CML production was increased slightly by adding only glucose, but notably by adding GO. In addition the localization of CML was observed by findings through immunohistochemistry and not observed in the default load of glucose and GO, and the accumulation of CML was increased. CML has been known to be found in the epidermal layer which has a comparatively rapid turnover in skin 7), and this report is not different from this finding in that CML was often observed in epidermis including the horny cell layer in the load of glucose and GO.

The histo-6luorescence AGEs, which was AGEs except for CML, increased as a result of glycation stress. A typical fluorescent AGEs is pentosidine (maximum wavelength: ex 335 nm, em 385 nm), but pentosidine in tissue was below detection sensitivity. Pentosidine is attracting attention as an early clinical marker of nephropathia and brittle-bone disease (bone aging) 8,9), and is known to be found in skin collagen and increased with aging 10). Pentosidine has strong NF-xB activating action and evokes inflammatory change to skin by the intermediary of inflammatory cytokine 7). Sell et al. showed that pentosidine is present in skin 11). But they used a sample including dermis, and it was not different from the three-dimensional human cultured epidermis used in this study. It was thought that pentosidine could be not measured because the skin model (MEL-300 three-dimensional human cultured epidermis) used in this study contained dermis.

The rest of the fluorescent AGEs are crossline (ex 379 nm, em 463 nm) 12) and pyrropyridine (ex 370 nm, em 455 nm) 13). It is necessary to investigate the localization of these fluorescent AGEs in tissue from now.

Skin has various proteins. Lysine and arginine residue-formed collagen protein are susceptible to glycation reaction, becoming AGES and forming interfibrinous cross-links, such that collagen looses mobility 3). The glycation of collagen is thought to be the cause of dermal sclerosis, wrinkling and the deterioration of the skin’s elasticity 14,15). We had confirmation that fluorescent AGEs were produced by incubating collagen, keratin, elastin and proteoglycan in the presence of glucose at 60°C for 10 days 16). The amount of CML was shown to be high in the reaction with keratin 16), and was consistent with immunohistochemical findings of the localization of CML being notably observed in the horny cell layer.

The three-dimensional human cultured epidermis used in this study was suitable as a glycation stress model in skin because CML, which was a non-fluorescent AGEs, and fluorescent AGEs were produced by adding GO to the medium and histological damage was observed. Especially the cumulative dosage of fluorescent AGEs could be optically quantitatively evaluated easily. It is thought that the inhibiting effect of the glycation reaction of anti-glycation substances will be examined using this model from now.

![Fig. 5. Fluorescence intensity value in 3-dimensional skin model using lamp B. Glc; glucose, GO; glyoxal.](image-url)
Conclusion

From this experiment, the skin glycation model based on normalization to CML production, texture observation, and AGEs fluorescence was made by adding glucose or GO to a three-dimensional human cultured epidermis. It’s hoped to undertake an investigation regarding the glycation reaction in skin developed using this experimental model from now.

Conflict of interest statement

The authors declare no financial or other conflicts of interest in the writing of this paper.

References