Glycative Stress Research

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Review article

Glycative stress and anti-aging: 3. The evaluation of glycative Stress: Measurement of advanced glycation end products (AGEs).

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Abstract

To evaluate glycative stress, a variety of substances produced during the process of glycation reaction can work as markers. There are various substances such as N^{ε}-carboxymethyl lysine (CML), pentosidine, and N^{ω}-carboxymethylarginine (CMA) which appear as advanced glycation end products (AGEs) that are produced in the end-stage of the glycation reaction. CML is an AGE that relates to the aging of skin. Pentosidine is a marker for early clinical stage nephropathy, and is known as a clinical marker which reveals the aging of bone quality. The HPLC and the ELISA are used to measure AGEs. When measuring AGEs in biospecimens, it is important to preprocess samples so that they will match the chemical characteristics of the AGEs. In particular, great care needs to be taken when providing heat treatment, because protein solutions that include glucose are apt to generate artificial AGEs. The cumulative dose of AGEs in skin can be measured non-invasively using the AGE Reader. Moreover, corneum CML can be measured using the horny cell layer collected by the tape stripping method. This constitutes a non-invasive measurement for AGEs.

***KEY WORDS*:** glycative stress marker, measurement method, advanced glycation end products (AGEs), tape stripping method, pentosidine

1. Measurement of AGEs

When evaluating glycative stress, a variety of substances produced during the non-enzymatic reaction of glucose and protein in the organism work as a glycative stress marker. Advanced glycation end products (AGEs) which are produced at the end of the glycation including N^{ε}-carboxymethyl lysine (CML) [1], pentosidine [2], N^{ω}-carboxymethylarginine (CMA) [3] and other various substances (***Fig. 1***).

CML is a non-fluorescent/non-crosslinking AGE produced from lysine using glyoxal (GO) as an intermediate for AGEs, which are also produced when patients suffer from diabetes or increasing oxidative stress. When collagen, which is transformed into CML, is added to the culture medium of fibroblast, apoptosis is induced [4]. It is included in the epidermides whose metabolic turnover is rapid compared to other layers [5]. Accumulation of CML in the corneum is related to the decline of the texture of the skin [6].

Pentosidine is a fluorescent and crosslinking AGE which is efficiently generated from ribose, arginine and lysine, and its medical fee is set as one of the early clinical markers of diabetic nephropathy. In recent years, pentosidine in the blood or urine has become increasingly utilized as a clinical marker that reveals the aging of bone quality, and is also expected to be used in the diagnosis of osteoporosis [7]. Furthermore, pentosidine is found in the collagen of skin and increases with age. Diabetes leads to a higher cumulative amount of pentosidine in the skin compared to normal subjects of the same age [8].

CMA is a kind of AGE which is generated from arginine using GO as a glycation intermediate, and exists specifically in collagen [3].

To perform a measurement of blood CML or pentosidine [9,10], the ELISA kit (enzyme-linked immunosorbent assay ; ELISA) has been commercialized by several Japanese and foreign companies. However, for the measurement of AGEs in biospecimens, a pretreatment of samples matching the chemical properties of the AGEs is very important. Great care must be taken not to provide heat treatments to blood samples carelessly when performing measurements of CML or pentosidine. This is because applying heat treatment to protein solutions including glucose necessarily bring about the generation of (artificial) AGEs. A heat treatment

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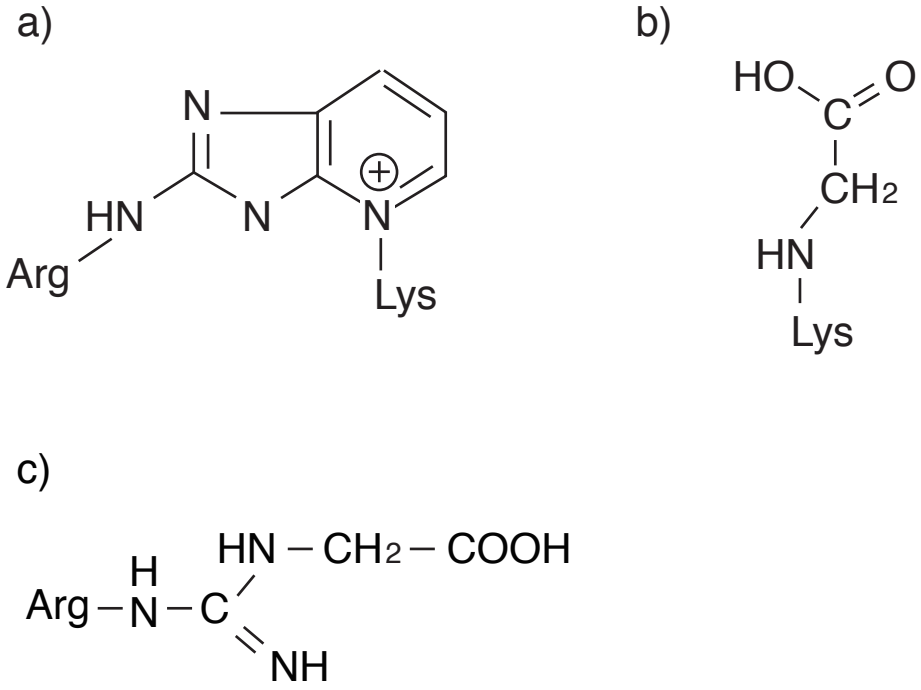

Fig. 1. **Chemical structure of pentosidine, CML and CMA.**
a) pentosidine, b) N^{ε}-carboxymethyl lysine (CML), c) N^{ω}-carboxymethylarginine (CMA).

of samples in a neutral condition may cause an error by causing measured values to be higher [11].

It is possible to prevent artificially high measurements of blood pentosidine by adding in a step of providing hydrolysis to samples by hydrochloric acid and a method of high performance liquid chromatography (HPLC) measurement [12,13]. The method in this study is based on the fact that glycation does not develop under strongly acidic conditions. Hydrolyzed blood samples remove impurities through ion-exchange columns. Pentosidine in samples hydrolyzed by the strong acid is to be assayed by reversed-phase HPLC (***Fig. 2***) [13]. In order to detect pentosidine, its unique fluorescent character (excitation wavelength: 335 nm, and fluorescent wavelength: 385 nm) is utilized. However, there are several problems with this method, such as a low recovery rate during pretreatment, the impossibility of removing impurities from the measurement peak, as well as difficulty measuring multi-specimens.

2. Non-invasive measurement of AGEs accumulated in skin using the AGE Reader®

The accumulation of AGEs in the skin is considered to be one of the causes of aging of the skin by lowering firmness or elasticity. It is possible to measure the accumulated AGEs in the skin non-invasively using the AGE Reader (DiagnOptics Technologies BV, Groningen, Netherlands). It can be measured based on auto-fluorescence (AF) [14,15]. The AGE Reader is a clinical measurement apparatus which was developed with the purpose of evaluating the progressive risk of complications of diabetes [16-18]. There are various types of AGE Readers which are used depending on the purpose of the attempted measurement. Each apparatus is meant to utilize the characteristics of fluorescent AGEs accumulated in tissues: they emit a peculiar auto-fluorescence by excitation, when ultraviolet light irradiates the skin. The AGE Reader SU which is meant to be used for research, has a light source (UV-A black-light) in its box-shaped body (280 mm: length x 150 mm: width x 115 mm: height). Lights with 345 ~ 410 nm wavelengths are to be irradiated through a 2 x 2 cm glass window onto the skin surface to cause excitation. Light with a fluorescence of 420 ~ 600 nm which is emitted from the skin by the irradiation is then introduced to the spectroscope so that the obtained information can be analyzed by special software for the calculation of AF [19]. The measurement time is about 90 seconds (in the case of three times measurements mode). The AGEs Reader measures the reflected light on the surface of the skin (reflection; Ref) by the white light-emitting diode (LED) light at the moment the fluorescence is emitted. If the Ref is below 12 %, the measurement value will be modified. Moreover, if the Ref is below 6 %, the measurement is rendered impossible. For this reason, when the skin color of subjects is dark brown or black (Fitzpatrick class 5-6 skin color) effective measurements cannot be conducted. It is necessary to clean the measurement spot sufficiently before measuring anything, because values are apt to be affected by the usage of skincare cosmetics including ultraviolet light absorbents.

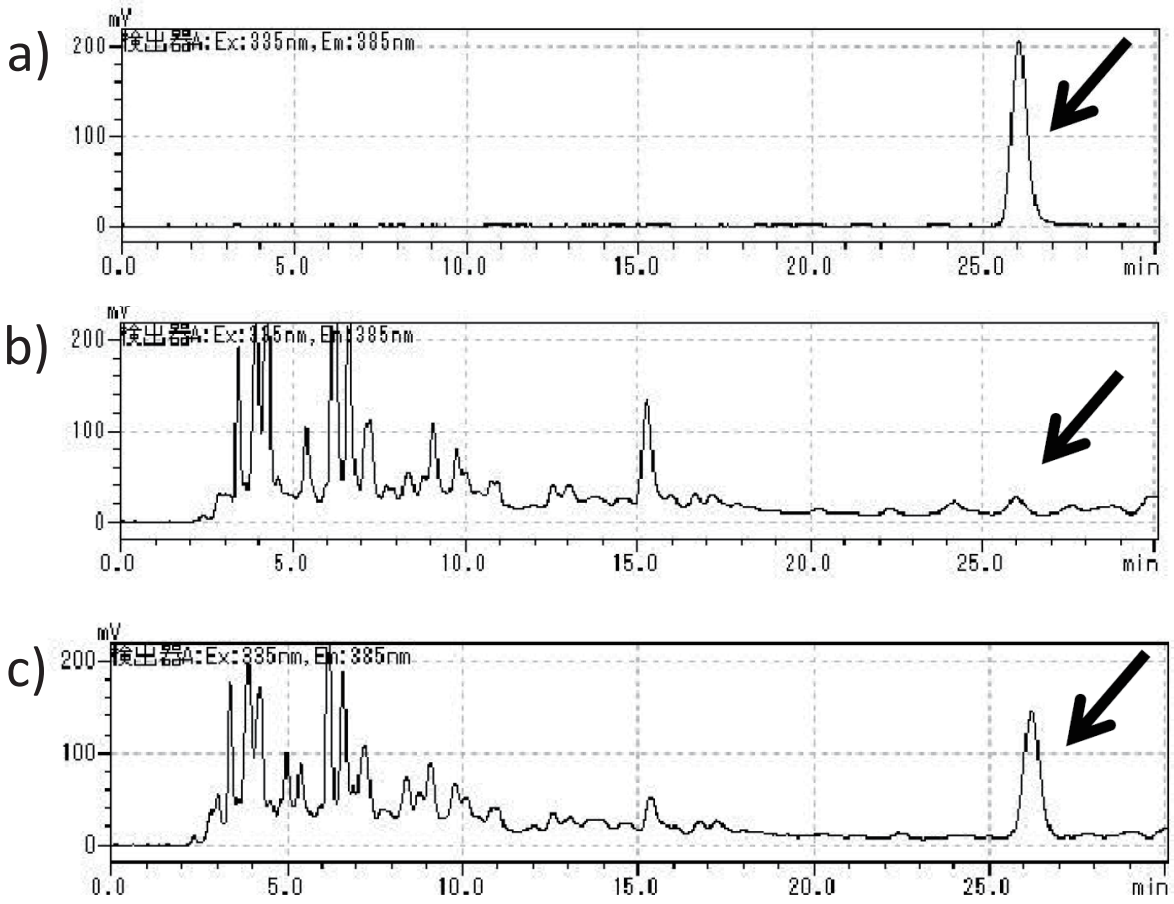

Fig. 2. **Serum pentosidine measurement by HPLC.**

HPLC condition: column, CAPCELL PAK C18 UG80 S-5 (Shiseido); eluent, a) 16% acetonitrile containing heptafluorobutyric acid (HFBA), b) 16% acetonitrile containing 0.26% HFBA, c) 60% acetonitrile containing 0.1% HFBA; Gradient, 0%B (0-25 min), 100%B (25-35 min), 0%B (35-45 min); flow rate, 1.0 mL/min; detection, fluorescent exctation 335 nm / emission 385 nm); arrow, pentosidine peak. a) pentosidine standard solution (527 fmol/μL), b) healthy person's serum, c) patient's serum of the nephrosis. (adapted from Ref 13)

Skin auto fluorescence (AF) is calculated using the strength of the fluorescence (420～600 nm) compared to that of the excitation light (300～420 nm) and is designated using an arbitrary unit (AU). AF calculated by the AGE Reader is output together with a comparison graph made of accumulated data by the manufacturers, taking into consideration the subjects' sex and age. However, the analytic formula of AF has not been disclosed by the manufacturers.

Regarding the AF values measured by the AGE Reader, a relation between diabetics/ healthy people and aging in the case of white humans (Fitzpatrick class 1-3 skin color) is shown. It is shown that AF values increase in corporation with the progress of aging or diabetes.

The measurement spot for skin AGEs recommended by manufacturers is the forearms. Taking measurements on the forearms seemed to cause little burden to the subjects. However, measurement values tended to be affected strongly by subjects' ultraviolet irradiation depending on the season. According to the comparison of results for measurement spots in the case of white humans, there is a remarkable correlation between the measurement values on the forearms or lower limbs and the progress rate of diabetes complications [20].

On the other hand, Japanese people tend to get sunburnt from exposure to ultraviolet radiation and come to have darker skin colors. For this reason, in the case of measuring Japanese subjects, there is little influence from ultraviolet radiation on the inside part of their forearms. It is possible to obtain stable data for AF measurement regarding areas 10 cm away from the edge of the right elbow [21,22]. The measurement areas using the AGE Reader need to be selected appropriately depending on the influence of ultraviolet radiation, colors of skin, and the race of subjects. The AGEs measurement apparatus has been recently under construction by multiple Japanese manufactures.

3. Measurement of corneum CML by using the tape stripping method

The corneum is located in the outmost layer of the skin and consists of the stratum corneum and intercellular material. The corneum keeps its balance due to regeneration and disjunction / separation from the surface of the stratum corneum. It is continually reconstructing itself. The corneum maintains biophylactic functions such as water retention and providing a barrier against infection. On the other hand, research on changes to skin due to aging are inclined to be conducted from the viewpoint of histology with only a few cases of using a simple measurement method based on biochemistry.

One of the methods of evaluating non-invasively the function of the corneum is the tape stripping method which exfoliates and gathers the corneum using an adhesive tape. Regarding the tape stripping method, a morphological evaluation method, which evaluates the number, and areas of keratinocytes exfoliated as well as cell morphology, and a measurement of the residual rate of the nucleus, are reported. In addition, measurement results for proteins [23], keratin [24], kathepsin L [25], and diadermic drugs [26] from the corneums gathered by the stripping method have been reported.

The measurement of corneum CML using the tape stripping method has an advantage in that it is possible to directly conduct a measurement easily by gathering protein samples of the skin through glycation stress [27]. The adhesive tape or the corneum checker, AST-01 (Asahi Biomed, Chiyoda-ku, Tokyo) is pressed onto the inner part of a subject's right arm by the fingers for five seconds, and then the corneum is gathered by peeling the tape from the skin. The adhesive agent on the sheet for collecting corneum samples is removed using a micro homogenizer in the buffer solution of Tris-HC1 (pH 7.5). Then, the corneum extract is retrieved. To conduct a measurement of CML in the extracting solution, a commercialized kit based on the ELISA is utilized. Along with this, the amount of protein in the corneum's protein extracts is measured, so that the CML amount per corneum protein is calculated. The corneum CML increases with age. Furthermore, skin with a high corneum CML rate has reduced elasticity (*Fig. 3*).

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Conflict of interest statement

There are no items deemed to be conflicts of interest in this research.

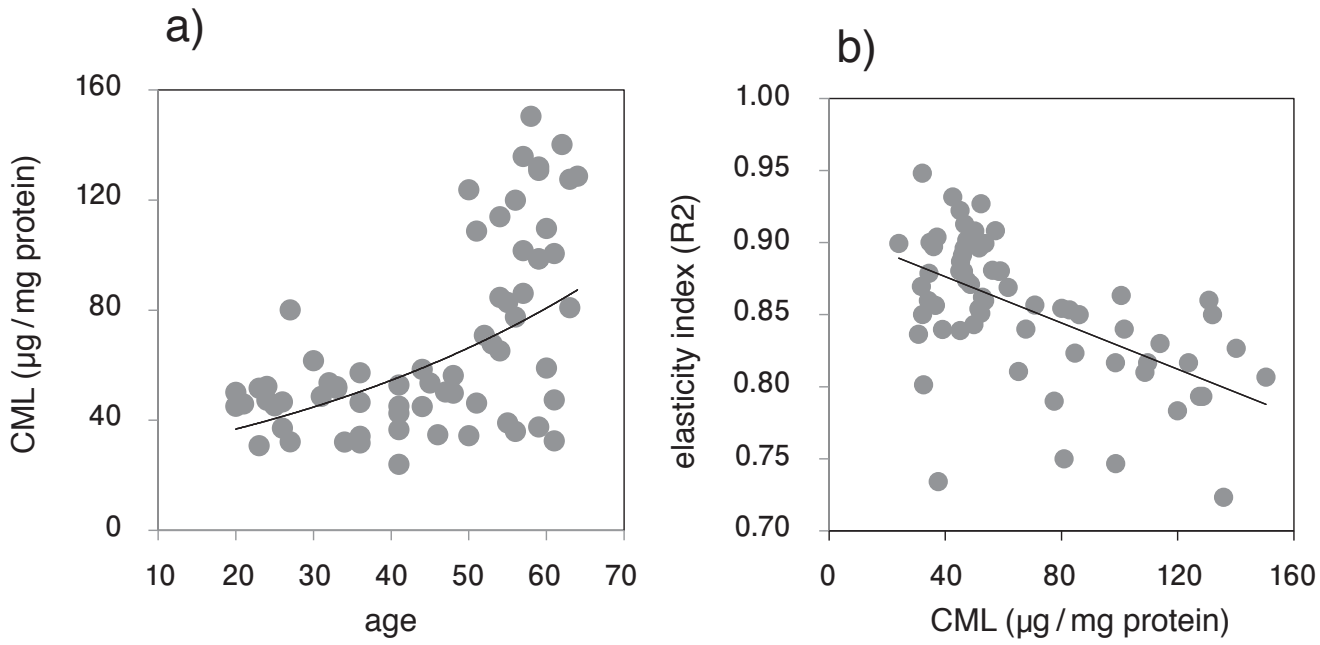

Fig. 3. **CML content in a corneum, and the relation of aging and skin elasticity.**

Healthy Japanese woman (n = 63), a) y = 24.85e0.0196x, r = 0.567, p < 0.01, b) y = -0.0008x + 0.9085, r = 0.569, p < 0.01. CML, N^{ε}-carboxymethyl lysine; elasticity index (R2), measured by Cutometer.

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