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Original article

Development of a prototype anti-glycation assay kit for assessment of bone and cartilage collagen modification

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Abstract

***Objectives*:** The currently available commercial collagen anti-glycation assay kits only use skin-derived collagen; none ofthem use bone-derived collagen. The use of bone-derived collagen for the assessment of glycation inhibition may contribute to the development of materials useful for the prevention of musculoskeletal disorders, such as reduced bone mineral density and reduced bone quality. The objective of this study was to develop a prototype of a novel collagen anti-glycation assay kit using bone- and cartilage-derived collagen and dentine slices.

***Methods*:** By using skin-, cartilage- and bone-derived collagen models (referred to as Skin-Col, Cartilage-Col and Bone-Col, respectively), the measurement of collagen concentration in each model sample, assessment of the degree of gelation (via absorbance measurement at 400 nm wavelength), and molecular structure analysis using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were performed. For glycation assay, after each sample was heated at 37°C overnight to gelate, different concentrations (0-20 mM) of glycation inhibitor aminoguanigine (AG) and 500 mM glyceraldehyde (GA) were added and incubated at 37°C for 24 hours to induce glycation. The glycated samples were subjected to measurement of the intensity of AGE-specific fluorescence (at 370 nm excitation and 440 nm emission). To evaluate the browning of bone quality assessment model samples, dentine slices were treated with GA (100-250 mM) and incubated at 37°C for 24 hours, followed by visual reading of browning.

***Results*:** The collagen concentration was the highest in the 1% Skin-Col solution and it was comparable between the 1% Cartilage-Col and concentrated Bone-Col solutions. Skin-Col showed the highest turbidity, followed in order by Bone-Col and Cartilage-Col, although the latter two were comparable. The molecular structure analysis revealed that Cartilage-Col contained more molecules of typical type 2 collagen comprising a single alpha-1 chain, than Skin-Col. Skin-Col and Cartilage-Col showed few bands in the low molecular ranges below the alpha chain, indicating that these samples contained few degradation products. Skin-Col showed the highest intensity of AGE-specific fluorescence, followed in order by Cartilage-Col and Bone-Col. All samples showed an AG concentration-dependent decrease in fluorescence intensity.

***Conclusion*:** These results demonstrate comparable properties of cartilage- and bone-derived collagen to that of skin-derived collagen and they suggest that these types of collagen can be used for the development of anti-glycation assay kits for the assessment of collagen modification in various parts of the body.

***KEY WORDS*:** Quantitative kit, glycation, bone, cartilage, collagen,

Introduction

Sugars are a family of nutrients essential for biological activities. However, when coexisting with biological proteins, sugars can modify and cross-link with lysine and arginine residues in a protein, thereby changing the three-dimensional structure of the protein and substantially altering its physical property as well as activity [1, 2]. This reaction, referred to as glycation or the Maillard reaction, consists of an early-stage reaction, where Amadori rearrangement products are generated, and a late-stage reaction, where these products are converted through oxidization, dehydration, condensation and other processes into advanced glycation endproducts (AGEs) [1, 2]. Collagen, which is a structural protein that helps form organs throughout the body, including skin [2, 3], bone [4, 5] and cartilage [6, 7], can also be modified by glycation.

The recent development of kits that can assess the degree

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of glycation of collagen [8] and elastin [9] in a cell-free and non-enzymatic manner, has facilitated the screening of substances that can inhibit glycation of collagen. These kits have been used for the assessment of anti-glycation materials in the development of functional foods and cosmetics. The currently available commercial collagen anti-glycation assay kits only use skin-derived collagen; none of them use bone-derived collagen. The use of bone-derived collagen for the assessment of glycation inhibition may contribute to the development of materials useful for the prevention of musculoskeletal disorders, such as reduced bone mineral density and reduced bone quality. The objective of this study was to develop a prototype of a novel collagen anti-glycation assay kit using bone- and cartilage-derived collagen and dentine slices.

Methods

Preparation of collagen solutions

The skin collagen model (Skin-Col) was prepared by mixing a 1% porcine skin-derived type 1 collagen solution (NH Foods Ltd., Osaka, Japan) with an equal volume of double-concentrated phosphate-buffered saline (PBS) and shaking at 4°C. The cartilage collagen model (Cartilage-Col) was prepared by adding 9.9 mL of ultrapure water to 0.1 g of a porcine cartilage-derived type 2 collagen lyophilizate (NH Foods Ltd.) and shaking at 4°C. The bone collagen model (Bone-Col) was prepared by adding 200 mL of ultrapure water to 2.2 g of a bovine bone-derived type 1 collagen lyophilizate (Cosmo Bio Co., Ltd., Tokyo, Japan) and shaking at 4°C. The solution was then centrifuged at 12,000 G and the supernatant was used as the sample solution.

Collagen concentration measurement

The collagen concentration of each sample was measured using a collagen assay kit (Collagen Quantitative Kit; Cosmo Bio Co., Ltd.).

Turbidity measurement

Each collagen sample was mixed with an equal volume of double-concentrated PBS, aliquoted into a 96-well black plate and allowed to gelate at 37°C under humid condition (***Fig. 1***). The degree of gelation (fibrillization of collagen) was assessed by absorbance measurement at 400 nm wavelength.

SDS-PAGE

Molecular structure analysis was performed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Each collagen sample (50 µL) was mixed with an equal volume of the double-concentrated sample buffer for SDS-PAGE, heated at 80°C for 2 minutes and used as a sample solution for electrophoresis. Each sample was loaded into wells of a 7.5% precast gel [MULTIGEL II mini 7.5 (13W); COSMO BIO Co., Ltd.] and electrophoresed at 30 mA for 55 minutes. The gel was then stained with Coomassie Brilliant Blue (CBB) solution for 1 hour and then decolorized overnight.

Glycation assay

To each sample, an equal volume of double-concentrated PBS was added under low temperature conditions to prepare a neutralized solution. This solution was aliquoted in 50-µL volumes into a 96-well black plate and heated at 37°C overnight to allow the collagen solution to gelate. To each well containing collagen gel, different concentrations (0-20 mM) of aminoguanigine (AG), a glycation inhibitor, and 500 mM glyceraldehyde (GA) were added and incubated at 37°C for 24 hours to induce glycation [10]. The degree of glycation was assessed by measuring the intensity of AGE-specific fluorescence (at 370 nm excitation and 440 nm emission) on a fluorescence plate reader (Infinite M200 PRO, Tecan Group Ltd., Männedorf, Switzerland) [10, 11].

Fig. 1. **Each sample of collagen gel.**

Preparation of a bone quality assessment model

Dentine slices (300 μm in thickness and 6 mm in length; Cosmo Bio Co., Ltd.) were placed into a 96 well black plate, to which PBS or AG (0-10 mM) and GA (100-250 mM) solutions were added. The plate was incubated at 37 °C for 24 hours to induce glycation. The degree of glycation was evaluated according to the browning of samples [12].

Results

Collagen concentration measurement

The collagen concentrations of Skin-Col and Cartilage-Col were 5.58 mg/mL and 3.73 mg/mL, respectively. The collagen concentration of Bone-Col was 0.2 mg/mL before concentration and 3.05 mg/mL after concentration (***Table 1***).

Collagen gel turbidity

The turbidity measurements of porcine skin-derived collagen, porcine cartilage-derived neutralized collagen and bovine bone-derived neutralized collagen gels were 0.459863 ± 0.017977, 0.086663 ± 0.008986 and 0.101388 ± 0.027583, respectively (***Fig. 2***).

SDS-PAGE analysis

Skin-Col showed 2 bands near 100 kDa and 1 band near 200 kDa while Cartilage-Col showed 1 band near 100 kDa (***Fig. 3***). Skin-Col and Cartilage-Col had no distinct band in the low molecular ranges below alpha chain. In contrast, Bone-Col was less soluble than the other types of collagen and the dissolved collagen showed some bands of degradation products in the low molecular ranges below alpha chain.

AGE-specific fluorescence intensity

GA-induced AGE-specific fluorescence was detected in not only Skin-Col, but also Cartilage-Col and Bone-Col, and it was suppressed in an AG concentration-dependent manner (***Fig. 4***). The fluorescence intensity was highest in Skin-Col and comparable between Cartilage-Col and Bone-Col.

Table 1. Collagen concentration in each sample

Collagen sample	[mg/mL]
Skin-Col	5.58
Cartilage-Col	3.37
Bone-Col	3.05

Skin-Col, porcine skin collagen type 1 model; Cartilage-Col, porcine cartilage collagen type 2 model; Bone-Col, bovine bone collagen type 1 model. Each sample was diluted 100-fold.

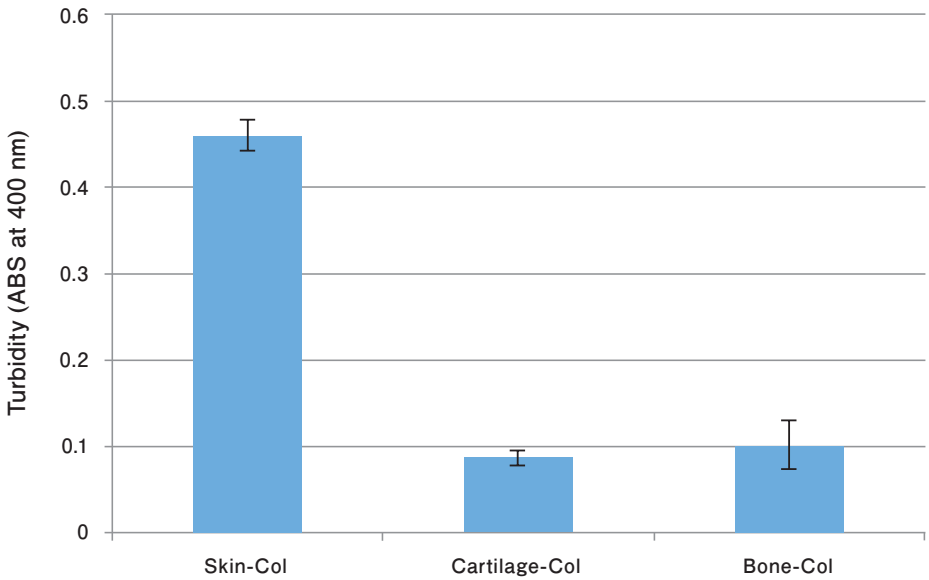

Fig. 2. **Turbidity of each collagen sample.**

Results are expressed as mean ± standard deviation, n = 3. Skin-Col, porcine skin collagen type 1 model; Cartilage-Col, porcine cartilage collagen type 2 model; Bone-Col, bovine bone collagen type 1 model.

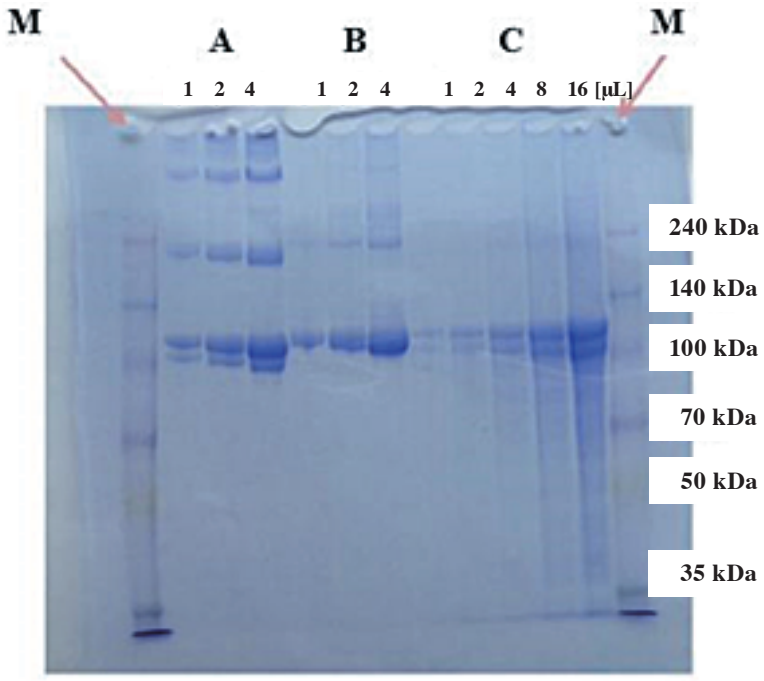

Fig. 3. **SDS-PAGE**

A: Skin-Col, B: Cartilage-Col, C: Bone-Col, M: molecular weight markers. SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Skin-Col, porcine skin collagen type 1 model; Cartilage-Col, porcine cartilage collagen type 2 model; Bone-Col, bovine bone collagen type 1 model.

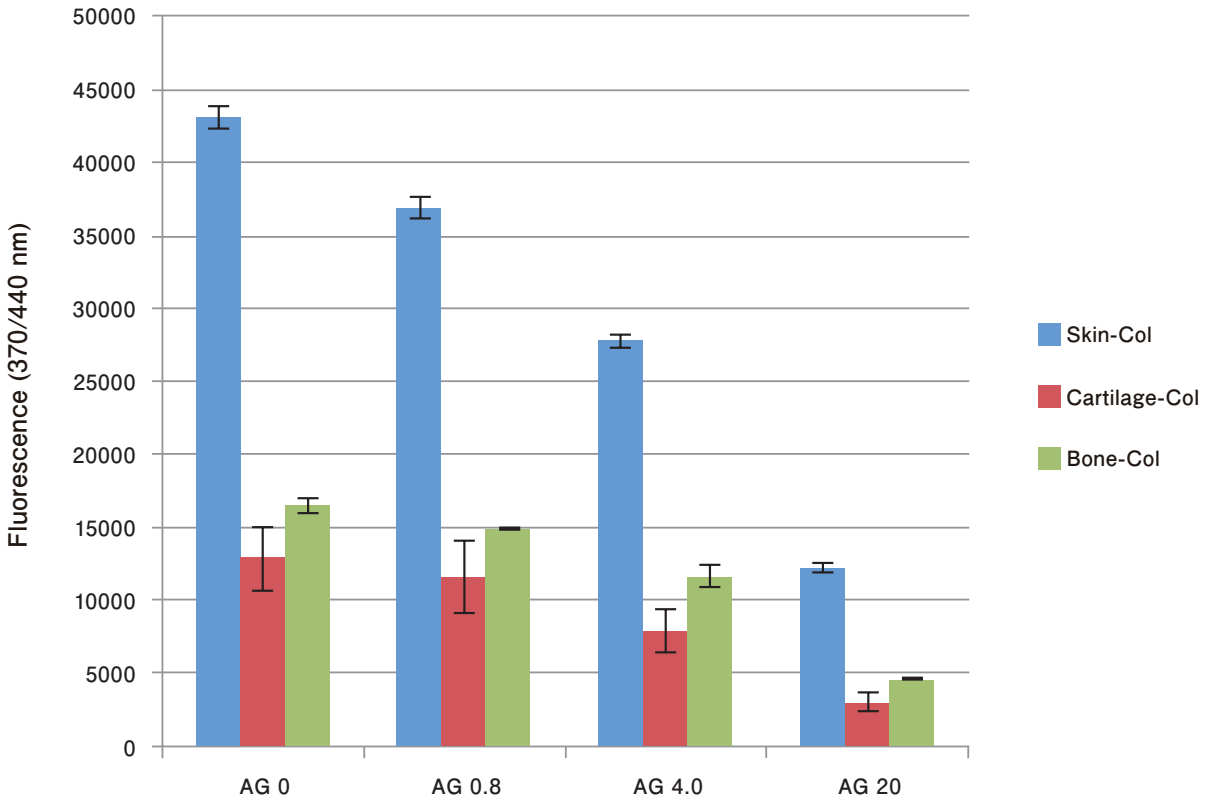

Fig. 4. **AGE fluorescence of each collagen sample.**

Results are expressed as mean ± standard deviation, n = 4. AGE, advanced glycation end products; AG, aminoguanidine (0 mM, 0.8 mM, 4.0 mM, 20 mM); Skin-Col, porcine skin collagen type 1 model; Cartilage-Col, porcine cartilage collagen type 2 model; Bone-Col, bovine bone collagen type 1 model.

Preparation of a bone quality assessment model

Dentine slices not treated with GA (0 mM) showed no browning while all samples treated with 250 mM GA showed browning, although their glycation was not inhibited by the addition of AG (***Fig. 5***). In contrast, in samples treated with 100 mM GA, browning was inhibited by AG in a concentration-dependent manner (***Fig. 6***).

Discussion

The objective of this study was to develop a novel collagen anti-glycation assay kit using bone- and cartilage-derived collagen and dentine slices. A prototype of the assay kit is shown in ***Fig. 7***.

Cartilage- and bone-derived collagen

In order to determine whether anti-glycation assay can be performed using Cartilage-Col and Bone-Col in the same manner as with Skin-Col, we performed collagen concentration and turbidity measurement, molecular structure analysis, and glycation assay to measure the intensity of AGE-specific fluorescence using the three collagen samples.

For collagen concentration measurement, we initially prepared Bone-Col as a 1% solution, the same concentration as the Skin-Col and Cartilage-Col solutions, but found that this sample contained a low concentration of soluble collagen (data not shown). We then prepared a concentrated solution of Bone-Col and confirmed that this sample contained a comparable amount of collagen to Cartilage-Col (***Table 1***). The highest turbidity was measured with Skin-Col, followed

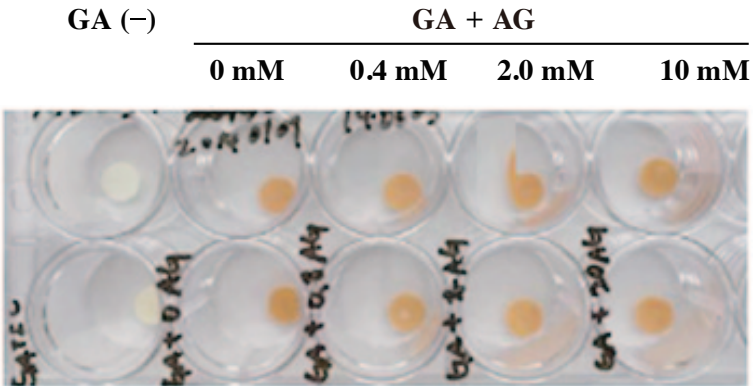

Fig. 5. **Browning of dentinal samples.**
GA, glyceraldehyde; AG, aminoguanidine (0~10 mM).

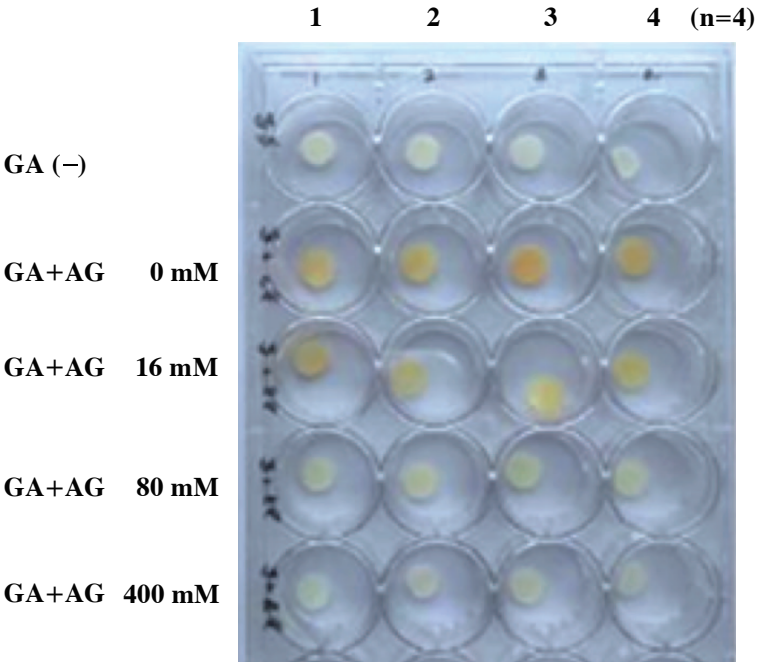

Fig. 6. **Browning of dentinal samples II.**
GA, glyceraldehyde; AG, aminoguanidine.

Fig. 7. **Prototype of Bone & Cartilage Glycation Assay Kit.**

in order by Bone-Col and Cartilage-Col, although the latter two were comparable (***Fig. 2***).

The molecular structure analysis revealed that Cartilage-Col contained more molecules of typical type 2 collagen comprising a single alpha-1 chain, as compared to Skin-Col (***Fig. 3***). Skin-Col and Cartilage-Col showed few bands in the low molecular ranges below the alpha-1 chain, indicating that these samples contained few degradation products. In contrast, bovine bone-derived collagen was less soluble than the other collagen samples and the scarce soluble collagen produced band-like degradation products in the low molecular ranges below the alpha-1 chain, suggesting that there are some cracks or other anomalies in the collagen molecule.

The highest intensity of AGE-specific fluorescence was measured with Skin-Col, followed in order by Cartilage-Col and Bone-Col (***Fig. 4***). All samples showed an AG concentration-dependent decrease in fluorescence intensity. This can be explained by the generation of more glycated proteins in samples with higher collagen concentrations [10, 11]. The differential fluorescence intensities among samples may be attributable to differential content and reactivity of glycation-susceptible sites in a collagen molecule, such as lysine and arginine. We have previously examined the correlation of AGE-specific fluorescence with the concentrations of lysine and arginine using human serum albumin (HSA), bovine serum albumin (BSA), type 1 collagen, keratin, elastin [13] and proteoglycan, and observed a higher intensity of AGE-specific fluorescence generated by the glycation of proteins containing more lysine [10]. Other possible reasons, such as differential tissue sources (type of collagen) and fibrillization (gelation) of collagen under neutralizing conditions, need to be addressed in future studies.

The anti-glycation effect of ingredients contained in tea/herbs [11], spices [14], vegetables [15] and fruits [16] have been examined using HSA. Collagen anti-glycation assay kits using skin-derived collagen have also been in practical use. The present results demonstrated comparable properties of cartilage- and bone-derived collagen to that of skin-derived collagen and suggest that these types of collagen can also be used for the assessment of anti-glycation activity of various substances, as performed in previous studies.

Bone quality assessment models

In an attempt to develop an assay kit that can visualize bone glycation, we treated dentine slices with GA and heated them to induce glycation, and found that the addition of glycation inhibitor AG inhibited the browning of samples in an AG concentration-dependent manner (***Figs. 5, 6***).

Studies have shown that aging and diabetes can induce browning of various parts of the body [17, 18]. In our previous studies, we measured the fluorescence spectra of a bovine skin-based glycation model and a human skin at an excitation wavelength of 370 nm and found that glycation and aging can induce increased intensity of AGE-specific fluorescence (at an emission wavelength of 444 nm) [19]. We have also reported a positive correlation of fluorescence intensity at wavelengths around 370 nm with calendar age, vascular age and poor lifestyle [20]. These findings suggest that the bone samples which showed browning in this study, can also be used in a visually readable anti-glycation assay kit. The dentine

slice samples used in this study have been used for evaluating osteoclast activity and also for other purposes and they can be a very unique experimental system that can be directly used for evaluating the effect of AGEs on osteoclasts.

Conclusion

The present results demonstrated comparable properties of cartilage- and bone-derived collagen to that of skin-derived collagen and suggest that these types of collagen can be used for the development of anti-glycation assay kits for the assessment of collagen modification in various parts of the body.

Acknowledgement

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Conflict of interest statement

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