

Original Article

***N*^ω-(carboxymethyl)arginine Accumulates in Glycated Collagen and Klotho-deficient Mouse Skin**Satoko Shimasaki¹⁾, Midori Kubota¹⁾, Makiko Yoshitomi²⁾, Kyoko Takagi³⁾, Kazuma Suda³⁾, Katsumi Mera²⁾, Yukio Fujiwara²⁾, Ryoji Nagai¹⁾

1) Department of Food and Nutrition, Laboratory of Biochemistry & Nutritional Science, Japan Women's University

2) Graduate School of Medical Sciences, Kumamoto University

3) Rohto Pharmaceutical Company Limited

Abstract

Objective: Advanced glycation end products (AGEs) accumulate in tissues due to aging, diabetic complications, and atherosclerosis. The acid lability of *N*^ω-carboxymethylarginine (CMA) present in glycated collagen has hampered detailed studies on its function and *in vivo* localization. In the present study, we analyzed the effects of collagen glycation on human dermal fibroblast (HDF) function. We also took advantage of Klotho-deficient mice (*kl/kl*), which undergo accelerated senescence, to determine glycated collagen's tissue localization.

Methods: Bovine type I collagen was incubated with ribose, and CMA formation was measured by enzyme-linked immunosorbent assay (ELISA). We measured the contraction of 3-dimensional matrix gels (3D gel), consisting of either native or glycated collagens, after culture with HDFs. CMA accumulation in Klotho-deficient mouse skin was measured by immunohistochemical staining.

Results: When collagen was incubated with ribose, CMA levels increased with time. In our HDF culture system, gels prepared with native, but not glycated collagen, contracted with time. In Klotho-deficient mice, CMA localized to the extracellular dermal matrix.

Conclusions: Here we show that CMA may provide a marker for collagen glycation, which may adversely affect HDFs' growth and survival. Therefore, treatment with AGE inhibitors might help prevent pathologies associated with AGE formation.

KEY WORDS: Advanced glycation end products (AGEs), Glycation, *N*^ε-(carboxymethyl)lysine (CML), Aging, Diabetic complications**Introduction**

Amino residues of proteins can react with reducing sugars via the Maillard reaction (glycation)¹⁾ leading to the formation of early intermediates such as Schiff bases and Amadori products, and subsequently to AGEs. AGE levels increase with aging^{2,3)}, diabetic complications⁴⁻⁸⁾, and atherosclerosis⁹⁻¹¹⁾. We previously demonstrated that GA-pyridine, which is generated from the myeloperoxidase system in activated leukocytes, accumulates in the cytoplasm of foam cells and extracellularly in the central region of atheromas in human atherosclerotic lesions. These results suggest that myeloperoxidase-mediated protein modification *via* glycolaldehyde may contribute to atherogenesis¹²⁾. Among AGE structures reported to date, *N*^ε-(carboxymethyl)lysine (CML), a major AGE antigenic determinant¹³⁾, accumulates in lens crystallins in an age-dependent manner¹⁴⁾. Pentosidine, one of the fluorescent AGE structures generated by oxidative conditions, accumulates in plasma and long-lived tissue proteins, such as the respective connective and lens tissue proteins collagen and crystallins. Its level reflects the extent of tissue damage associated with age-related diseases, such as diabetes mellitus, atherosclerosis, and chronic renal failure¹⁵⁾. Plasma pentosidine levels increase

in kidney failure¹⁶⁾. *N*^ω-carboxymethylarginine (CMA) (**Fig. 5**), an AGE component was identified in glycated collagen¹⁷⁾, suggesting that CMA may provide a marker for collagen glycation. CML is detected in many proteins such as collagen and albumin. However, nothing is known about CMA modification of tissue proteins. Because AGEs accumulate in collagen as a function of aging^{18,19)}, CMA may be involved in aging of collagen-rich tissues such as skin.

Skin consists of three layers as follow: epidermis, dermis, and subcutis. Dermal fibroblasts secrete extracellular matrix collagens types I and III into the dermis and proliferate by adhering to the resulting collagen matrix. These cells also secrete elastin and hyaluronic acid, both of which are important to maintain skin elasticity and hydration. Therefore, normal dermal fibroblast homeostasis contributes to the maintenance of healthy skin.

Alikhani *et al.*²⁰⁾ demonstrated that CML accumulation in collagen induced dermal fibroblasts to undergo apoptosis. They also reported that CML-modified collagen induced osteoblast apoptosis, which was mediated by the MAP kinase and cytosolic apoptotic pathways²¹⁾. Thus, collagen modification by AGEs may inhibit mitosis, induce apoptosis or premature senescence, thereby adversely affecting the normal functions of diverse collagen-expressing cell types.

Here we determined the effects of CMA formation on the contraction of a 3D gel when incubated with HDFs in culture. In order to investigate whether CMA accumulation increases with aging, we determined its accumulation rate in the skin of Klotho-deficient mice, a strain that senesces prematurely and exhibits multiple age-associated disorders such as skin obsolescence²².

Materials and Methods

Materials

The materials used in this study and their sources were as follows: bovine collagen (type I), Nitta Gelatin Co. (Osaka-city, Osaka Japan); gelatin and human serum albumin (HSA), Sigma Aldrich Japan (Shinagawa-ku, Tokyo Japan); horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody, Kirkegaard Perry Laboratories (Gaithersburg, MD, USA); streptavidin-alkaline phosphatase, Invitrogen (Carlsbad, CA, USA); goat serum, Dako (Glostrup, Denmark); and HistoMark RED kit, Kirkegaard & Perry Laboratories, Inc. (Washington DC, USA); D(-) ribose, Kanto Chemical Co. (Chuo-ku, Tokyo Japan); *O*-phenylenediamine dihydrochloride (OPD) tablets and hydrogen peroxide, Wako Pure Chemical Industries, Ltd. (Osaka-city, Osaka Japan); sulfuric acid, Wako Pure Chemical Industries, Ltd.

Anti-CMA Monoclonal Antibody

A monoclonal anti-CMA antibody²³ was labeled with biotin using a biotin Labeling Kit-NH₂ (Dojindo Laboratories, Kamimashiki-gun, Kumamoto Japan).

Animals

Eight 8 or 12 week-old female Klotho-deficient mice (Clea Japan Inc., Meguro-ku, Tokyo Japan)²² fed with a solid were divided into two groups of 4 animals: Group 1 (8 weeks old), Group 2 (12 weeks old), as approved by Rohto Pharmaceutical Company Limited. Briefly, the animals were housed in a pathogen-free barrier facility (12 h/12 h light/dark cycle), room temperature 23±2°C, humidity 55±15%, and were fed a normal rodent chow diet (CE-2, Clea, Japan). Mice were killed under pentobarbital anesthesia.

In vitro protein glycation

Ribose-HSA and ribose-gelatin were prepared by incubating 4 mg/mL each of HSA or gelatin with 60 mM ribose in PBS at 37°C for up to 7 days. Ribose-collagen was prepared by incubating 1.5 mg/mL collagen with 30 mM ribose in 200 mM phosphate buffer (pH 7.2) at 37°C for 7 days under sterile conditions. Collagen incubated alone in 200 mM phosphate buffer (pH 7.2) at 37°C for 7 days was used as a control.

ELISA

ELISA was performed as described¹². Briefly, each well of a 96-well microtiter plate was coated with 100 µL of collagen in PBS at the indicated concentration and incubated for 2 h. The wells were washed 3-times with PBS containing 0.05% Tween 20 (washing buffer). The wells were then blocked with

0.5% gelatin in PBS for 1 hr. After 3 washes, the wells were incubated for 1 h with 100 µL of an anti-CMA monoclonal antibody solution (1 µg/mL). Wells were washed 3 times, incubated with HRP-conjugated anti-mouse IgG, and then with 1,2-phenylenediamine dihydrochloride. The reaction was terminated with 100 µL 1.0 M sulfuric acid, and the absorbance was measured at 492 nm using a micro-ELISA plate reader.

Contraction capacity

The 3D gel culture system for HDFs was prepared using a collagen gel culturing kit (Nitta Gelatin Co.) according to the manufacturer's instructions. The 3D gels were prepared by embedding HDFs (12.5×10⁴ cells) in 0.4 mL collagen matrix in 12-well cell culture plates. The gels were covered with 1.7 mL Dulbecco's modified minimal medium and incubated for 2 days at 37°C in a 5% CO₂ incubator. To examine cell-mediated contraction activity, gels were detached from the rim of the wells and incubated further, up to 7 days. Diameters were measured on days 1 and 7. This collagen lattice assay system is based on experiments by Bell *et al.*²⁴. Delvoye *et al.*²⁵ describes the technique for measuring traction forces developed by cells embedded in a 3D collagen lattice.

Detection of caspase-3

The collagen matrix-containing HDFs were fixed with 2% paraformaldehyde and incubated with an anti-cleaved caspase-3 antibody (Asp175, Cell Signaling). After the removal of excess antibody by washing with PBS, the samples were incubated with a HRP-labeled sheep anti-rabbit antibody (Nichirei Corporation, Chuo-ku, Tokyo Japan). The reaction was visualized by using a 3,3'-diaminobenzidine tetrahydrochloride (Dojindo) substrate as described previously²⁶.

Tissues and Immunohistochemistry

Skin samples harvested from Klotho-deficient mice²⁷ were immediately frozen in liquid nitrogen and kept at -80°C until sectioned. Frozen tissue sections (8 µm) were air-dried and stored at -80°C. Thawed sections were fixed for 1 min in 100% ethanol at room temperature (RT), rinsed with Tris-HCl Buffer (0.05 M, pH 7.5), and blocked for 1 h at RT with 10 % goat serum. After washing thoroughly, the sections were incubated with biotinylated anti-CMA antibody for 1 h at RT. The sections were rinsed, incubated with streptavidin-conjugated alkaline phosphatase for 1 h at RT, rinsed, and stained using the HistoMark RED system, counterstained with the HistoMark RED system Contrast Blue Solution, and mounted. The stained sections were observed and photographed using an Olympus IX71 microscope.

Result

CMA content in Ribose-Gelatin and Ribose-HSA

CMA levels in ribose-gelatin and ribose-HSA were determined by ELISA. Gelatin's CMA content increased with time during incubation with ribose, whereas CMA levels in ribose-HSA were much less than those in ribose-gelatin (**Fig. 1**).

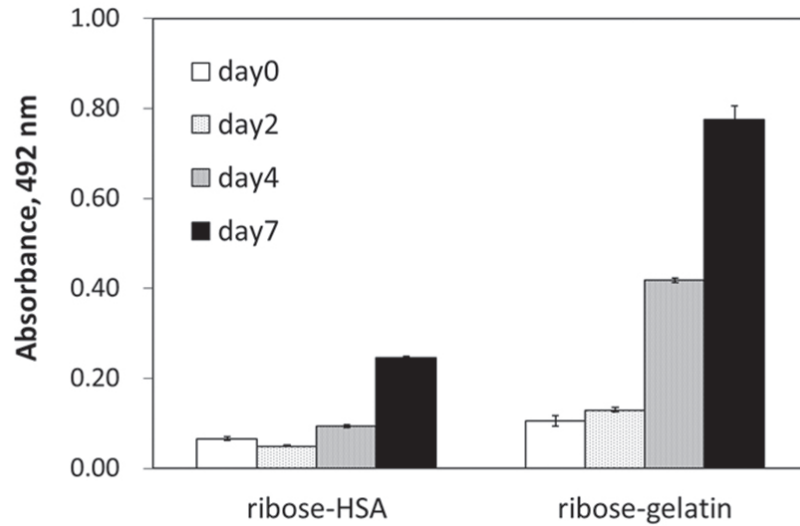


Fig. 1. Contents of CMA in ribose-gelatin and ribose-HSA. Ribose-HSA and ribose-gelatin were incubated at 37 °C for up to 7 days, and then CMA contents were measured by ELISA (n=3) as described in Materials and Methods.

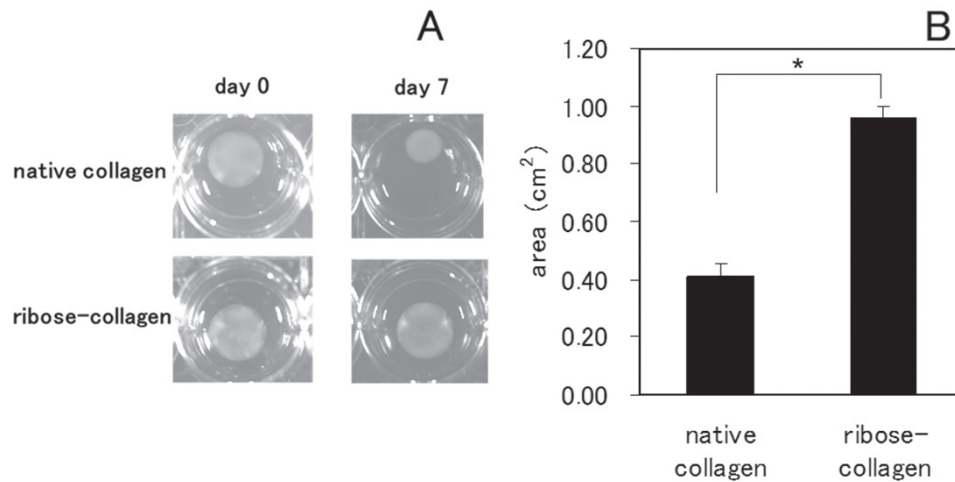


Fig. 2. Influence of ribose-collagen on the contractile capacity of fibroblasts. (A) HDFs were incubated for 7 days with control or ribose-collagens in 12-well cell culture plates. (B) The areas of 3D gels were measured after 7 days incubation. Data are presented as means ± SD. * $p < 0.001$.

Influence of Ribose-Collagen on Fibroblasts' Contractile Capacity

The areas of 3D-control or ribose-collagen gels were measured after 7 days culture with HDFs. The 3D matrix prepared with the collagen control contracted during a 7 day incubation (Fig. 2A). In contrast, ribose-collagen contracted much less ($p < 0.001$) (Fig. 2B). This result suggested that collagen glycation significantly diminished the ability of HDFs to infiltrate and contract the collagen gel.

Detection of caspase-3

We next compared the expression of cleaved caspase-3 in HDFs which were incubated with native collagen and glycated collagen. As a result, cleaved caspase-3 immunoreactive cells were observed only when HDFs were incubated with glycated collagen, thus suggesting that glycated collagen induces apoptosis in HDFs (Fig. 3).

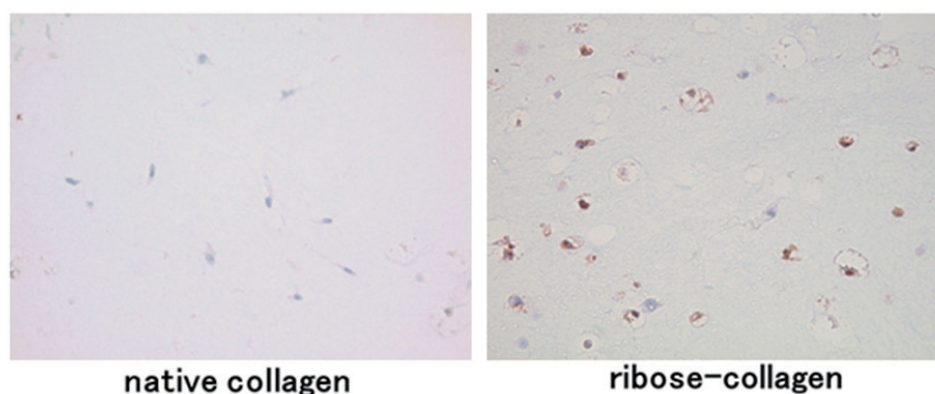


Fig. 3. Detection of cleaved caspase-3.

HDF was incubated for 7 days with control or ribose-collagens in 12-well cell culture plates. The collagen matrix-containing HDFs were fixed with 2% paraformaldehyde and incubated with an anti-cleaved caspase-3 antibody. After the removal of excess antibody by washing with PBS, the samples were incubated with a HRP-labeled sheep anti-rabbit antibody as described in Materials and Methods.

Immunohistochemistry

In order to evaluate CMA formation *in vivo*, we next measured CMA accumulation in Klotho-deficient mouse skin using an anti-CMA monoclonal antibody. Positive CMA immunoreactivity, indicated by red staining, was observed to accumulate in collagen-rich regions such as dermis (arrow). These data correlated well with *in vitro* observations. Furthermore, CMA accumulated in greater amounts in group 2 (12 weeks old) mice, as compared with group 1 (8 weeks old) mice. As a negative control, the same procedure was performed, but the first antibody was omitted. Non-immune mouse IgG was also used as a negative control, and the staining results showed no immunoreaction.

Discussion

More than forty types of AGE structures have been reported by investigators using a variety of analytical techniques such as fluorescence intensity, cross-linking, and resistance to acid hydrolysis¹⁾. Our goal was to provide a more consistent, specific, and convenient detection method by taking advantage of specific AGE antibodies, which can estimate AGE levels and their tissue localizations. CMA was originally identified in glycated collagen by enzymatic digestion¹⁷⁾. However, CMA's acid lability makes its detection by HPLC difficult when proteins are acid hydrolyzed. We found here that an anti-CMA monoclonal antibody specifically and sensitively detects CMA in collagen. Our immunochemical analyses were consistent with findings of other laboratories (Fig. 1).

In the present study, we estimated AGE-modification by measuring CMA in glycated collagen. HDF-mediated contraction of the 3D gel matrix was time-dependent, but significantly inhibited by CMA-collagen. Although several glycated collagen AGE structures have been identified²⁸⁾, CMA may provide a potential marker for collagen glycation. We also observed CMA accumulation in Klotho-deficient mouse skin

(Fig. 4), demonstrating that CMA formation occurs not only *in vitro* but also *in vivo*. It is known that fibroblasts adhere to a collagen matrix through cell membrane-localized integrin molecules. Integrins serve as cell adhesion molecules that recognize specific sequences such as RGD²⁹⁾ in collagen. Taken together, our results suggest that integrin binding domains such as the RGD motif may be modified by CMA, thereby altering dermal fibroblast function such as collagen secretion (Fig. 5).

Protein modification by AGEs progresses by reaction with glucose as well as with reactive aldehydes. For instance, glyoxal, methylglyoxal, glycolaldehyde, glyceraldehydes, glucosone, and 3-deoxyglucosone are generated by glucose oxidation and by enzymatic pathways involved in mediating inflammation and glycolysis (Fig. 5)³⁰⁾. Furthermore, modification of proteins with reactive aldehydes increases their net negative charge followed by induction of denaturation and loss of function³¹⁾. The acid stability of AGEs such as CML and pentosidine have enabled their structures to be defined in detail. Furthermore, pentosidine fluoresces when it cross-links lysine and arginine residues in proteins¹⁵⁾.

Based on the results of our present study and research by others we believe that additional studies are required to clarify the role of CMA-modified collagen on skin aging and the mechanisms by which CMA affects fibroblast functions and viability. If it becomes clear that CMA accelerates skin aging, screening for and identification of compounds that inhibit CMA formation will be important for combating and delaying skin aging.

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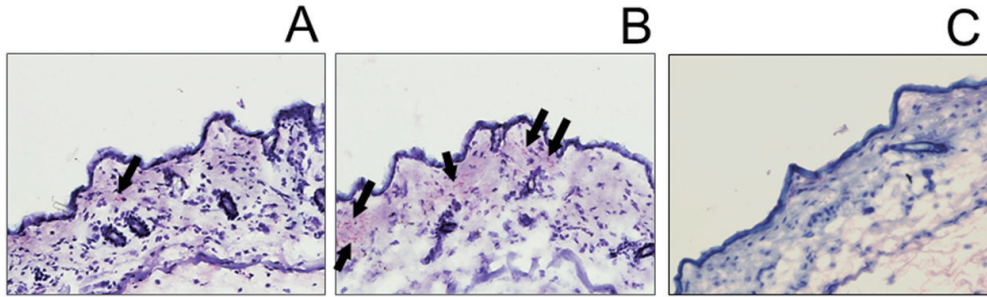


Fig. 4. CMA accumulation in the skin of Klotho-deficient mice.

Klotho-deficient mice at (A) 8 weeks, and (B) 12 weeks of age were used in this study. Red staining indicates CMA immunoreactivity. Non-immune mouse IgG was also used as a negative control, and the staining results showed no immunoreactions (C).

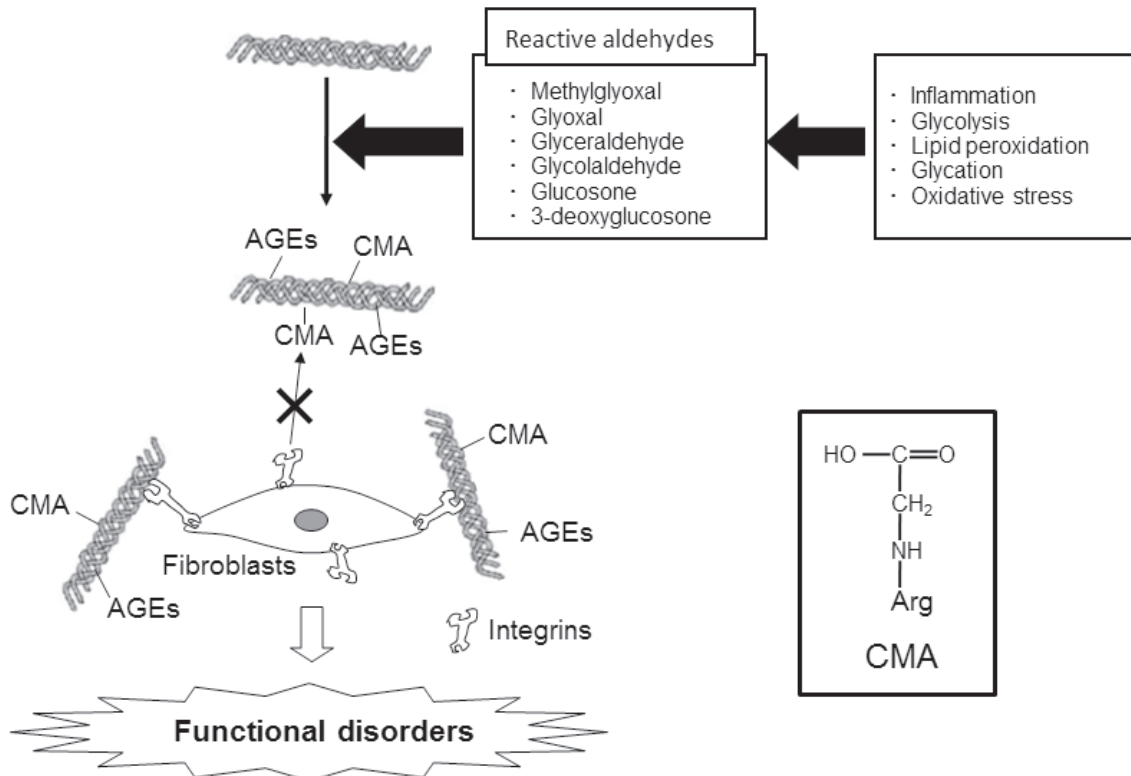


Fig. 5. Glycation-induced functional disorders of dermal fibroblasts.

Reactive aldehydes are generated from glucose oxidation by enzymatic pathways that mediate inflammation and glycolysis. The collagen binding domains of integrins may be modified by CMA, potentially adversely affecting dermal fibroblast function.

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