

Original Article

Establishment of a model for evaluating tumor necrosis factor- α production by cultured RAW264.7 in response to glycation stress

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Abstract

Objective: While extensive studies have been carried out on the glycation reaction *in vivo*, only a few studies have been conducted in cell culture systems. The objective of this study was to establish a model for evaluating the inflammatory reaction in response to glycation stress using macrophage-like cell line RAW264.7 to assess the relationship between advanced glycation endproducts (AGEs) and tumor necrosis factor- α (TNF α).

Methods: The inflammatory reaction induced by AGEs was quantified by measuring TNF α production by ELISA. Seven AGEs and glycation intermediates, including pentosidine (PEN), *N*^ε-carboxymethyl-L-lysine (CML), CML-modified human serum albumin (CML-HSA), *N*^ω-carboxyethyllysine (CEL), 3-deoxyglucosone (3DG), glyoxal (GO) and methylglyoxal (MGO) and three AGE-modified proteins (HSA, keratin and collagen-peptide) were applied to culture media of RAW264.7 cells. Each AGE-modified protein was incubated in phosphate buffer solution containing GO at 37°C for 2 days and measured for the content of CML and fluorescent AGEs. Concurrently with TNF α , the content of soluble receptor for AGEs (sRAGE) capturing AGEs outside the cell was measured by ELISA.

Results: TNF α production increased in a dose-dependent manner after the application of CML-HSA, a protein-bound AGE, but not with free AGEs (PEN, CML and CEL) or glycation intermediates (3DG, GO and MGO). TNF α production in response to AGE-modified proteins varied depending on protein type and it was reciprocally correlated with sRAGE production. No correlation was observed between TNF α production and the content of CML or fluorescent AGEs in the AGE-modified proteins.

Discussion: AGEs induced TNF α more rapidly than existing inflammation-inducing substances through RAGE and TLR4. TNF α activity increased at a concentration of CML-HSA similar to that in the human body suggests the potential for an AGE-induced inflammatory reaction *in vivo*. The results also suggest that collagen-peptide indirectly increases sRAGE production by activating matrix metalloproteinase.

Conclusion: We established a model for evaluating TNF α in response to glycation stress using RAW264.7 cell culture system. The results suggest that the tissue accumulation of AGEs may be responsible for the development of macrophage-mediated inflammation.

KEY WORDS: advanced glycation endproducts (AGEs), RAW264.7, tumor necrosis factor alpha (TNF α), soluble receptor for AGEs (sRAGE)

Introduction

An *in vivo* non-enzymatic reaction between reducing sugars, such as glucose, and a protein results in irreversible degradation of the protein. This reaction is referred to as glycation. Glycation consists of an early reaction, intermediate formation and late reaction. The early reaction is composed of non-enzymatic reactions between glucose and amino acids, resulting in the production of Schiff bases and eventually Amadori compounds. In the intermediate formation phase, intermediates containing a dicarbonyl group, such as 3-deoxyglucosone (3DG), glyoxal (GO) and methylglyoxal (MGO), are produced. In the late reaction phase, the dehydration and condensation of intermediates and Amadori compounds result in the production of advanced glycation endproducts (AGEs), such as pentosidine (PEN), *N*^ε-carboxymethyl-L-lysine (CML) and *N*^ω-carboxyethyllysine (CEL). Biological stress resulting from AGE production is

referred to as glycation stress, which is a known aging factor. Various *in vivo* characteristics of AGEs have been reported, such as the characteristics that AGEs accumulate not only in lesions of diabetic complications, but also in alveolar macrophages in patients with pulmonary fibrosis,¹⁾ and that CML is present in the epidermal layer of the skin, a tissue with a high turnover rate²⁾.

Macrophages express AGE-recognizing receptors, known as receptor for AGEs (RAGE), on their cell membrane. RAGE is composed of extracellular, transmembrane and intracellular domains. Various molecular species of RAGE are produced from a single gene through alternative splicing. An intracellular domain-deficient form of RAGE or cleavage of full-length transmembrane form of RAGE by matrix metalloproteinase (MMP) on the cell membrane results in the production of soluble RAGE (sRAGE)³⁾. sRAGE functions as a decoy receptor that inhibits the interaction between AGEs and RAGE by capturing AGEs outside the cell. A typical intracellular signaling pathway

of RAGE is the activation of transcription factor NF- κ B via ras/MAP kinase⁴). In the vascular endothelial cell, RAGE signaling has been shown to be involved in the local inflammatory reaction by inducing the expression of vascular cell adhesion molecule-1 (VCAM-1)⁵). In relation to inflammation, tumor necrosis factor- α (TNF α) is a predominant pro-inflammatory cytokine that is produced in the early stage of inflammation and induces endothelial cells to express leukocyte chemotactic factors.

Most studies on *in vivo* glycation reactions have been performed using *in vitro* systems. The establishment of a cell culture system to investigate glycation reactions is an important step for studying this process in animal models that more precisely reflect actual biological conditions. The objective of this study was to establish a model for evaluating TNF α production in response to glycation stress using macrophage-like cell line RAW264.7 to assess the relationship between AGEs and inflammation.

Methods

Evaluation of AGE-induced TNF α

Mouse macrophage-like cell line RAW264.7 was used in this study. With the evaluation of TNF α , previous reports involving RAW264.7 cells were reviewed to determine the cell density during subculture, CO₂ reaction time and the method for preparing AGE samples^{6,7}).

Cell culture

RAW264.7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10,000 units/mL penicillin, 10 mg/mL streptomycin, 25 μ g/mL amphotericin B and 10% FBS (Nihon Biosciences Inc., Tokyo, Japan) at 37°C and 5% CO₂. A cell suspension was prepared at a concentration of 5×10^5 cells/mL and added to a 24-well plate dish. After cells were cultured under the above condition for 24 hours, AGEs, glycation intermediates and AGE-modified proteins, which had been sterilized by filtration (New Steradisk, Kurabo Industries, Chuo-ku, Osaka, Japan), were added to the culture. Then, the culture supernatant was collected and used as a sample for the quantification of TNF α and sRAGE by enzyme-linked immunosorbent assay (ELISA). In these assays, lipopolysaccharide (LPS; Wako Pure Chemical Industries, Osaka, Japan) was used as the positive control.

Preparation of AGEs, glycation intermediates and AGE-modified proteins

Pentosidine TFA salt, CML, CEL (PPL-FR, Strasbourg, France), 3DG (Dojindo, Kumamoto, Japan), 40% glyoxal solution (Wako), methylglyoxal solution (Sigma-Aldrich) and CML-HSA (CircuLex, Nagano, Japan) were used as AGE samples. Each sample was diluted to 0.01-2.5 μ g/mL with DMEM.

AGE-modified proteins were prepared using human serum albumin (HSA; Sigma-Aldrich), keratin (wool-derived, code 09378-52; Nacalai Tesque, Kyoto, Japan) and collagen-peptide (cowhide-derived, code AFC-G; Nippi Inc., Tokyo, Japan). Three mg/mL solutions of each protein, 20 mmol/L solution of GO and 100 mmol/L of phosphate buffer solution (pH 7.4) were prepared, mixed at the volume ratios given in [Table 1](#) and allowed to react at 37°C for 2 days. The reacted samples were diluted to 1:5 with DMEM. GO-containing samples (GO2+) were prepared at different molecular weights (<10 K, 10-30 K and >30 K) by ultrafiltration using an Ultra Free MC filter unit (10,000

Table 1. Mixing ratios for preparing AGE-modified proteins

	GO (2+)	GO (2-)
Solutions of each protein (3 mg/mL)	500 μ L	500 μ L
Phosphate buffer solution (100 mmol/L)	100 μ L	100 μ L
GO (20 mmol/L)	100 μ L	0 μ L
Distilled water	300 μ L	400 μ L
Total	1,000 μ L	1,000 μ L

NMWL, 30,000 NMWL; Millipore, Billerica, MA, USA). All samples were sterilized by filtration and added to cell cultures. Concurrently, the content of CML and fluorescent AGEs in the reacted samples were measured as described below.

Quantification of TNF α

TNF α was quantified using a Murine TNF α ELISA kit (Diacclone, Besançon, France). Cell culture supernatant (n = 3) diluted to the optimal concentration with the standard diluent buffer was added to wells of the ELISA plate on which anti-mouse TNF α antibody was immobilized and incubated at ambient temperature for 2 hours. After wells were washed with the washing buffer provided with the kit, biotin-conjugated anti-mouse TNF α antibody was added and allowed to react at ambient temperature for 1 hour. After wells were washed with the washing buffer, streptavidin-HRP was added and allowed to sit at ambient temperature for 30 minutes. After wells were washed, tetramethylbenzidine was added and incubated for 30 minutes to develop color. After the reaction was stopped, the absorbance at 450 nm was measured with a microplate reader (SpectraMax Paradigm Multi-Mode Detection Platform, Molecular-Devices Japan, Tokyo, Japan). TNF α concentration was calculated from the absorbance measurements using a calibration curve created with known concentrations (31.25-1,000 pg/mL) of recombinant mouse TNF α .

Quantification of CML

CML was quantified using a CircuLex CML / N ϵ - (carboxymethyl) lysine ELISA kit (CircuLex, Ina, Nagano, Japan). Cell culture supernatant (n = 3) mixed with anti-CML monoclonal antibody MK-5A10 was added to wells of the ELISA plate on which anti-CML-BSA antibody was immobilized and allowed to react at ambient temperature for 1 hour. After wells were washed with the washing buffer provided with the kit, HRP-conjugated detection antibody was added and allowed to react at ambient temperature for 1 hour. After wells were washed with the washing buffer, tetramethylbenzidine was added and incubated for 10 minutes to develop color. After the reaction was stopped, the absorbance at 450 nm was measured with the SpectraMax Paradigm Multi-Mode Detection Platform (Molecular-Devices Japan). The CML concentration was calculated from the absorbance measurements using a calibration curve created with known concentrations (0.109-14 μ g/mL) of CML-HSA.

Quantification of fluorescent AGEs

The fluorescence intensity of AGEs was measured with the SpectraMax Paradigm Multi-Mode Detection Platform (Molecular-Devices Japan) at an excitation wavelength of 370 nm and fluorescence wavelength of 440 nm (n = 3). Each fluorescence value was calculated as a relative value to that of 5 μ g/ml quinine sulfate in 0.1 N sulfuric acid aqueous solution defined as 1,000.

Quantification of sRAGE

sRAGE was quantified with a RAGE Mouse ELISA kit (Abcam, Cambridge, UK). Cell culture supernatant ($n = 3$) was added to wells of the ELISA plate on which anti-mouse RAGE antibody was immobilized and incubated at ambient temperature for 2.5 hours. After wells were washed with the washing buffer provided with the kit, biotin-conjugated anti-mouse RAGE antibody was added and allowed to react at ambient temperature for 1 hour. After wells were washed with the washing buffer, streptavidin-HRP was added and allowed to react at ambient temperature for 45 minutes. After the wells were washed, tetramethylbenzidine was added and incubated for 30 minutes to develop color. After the reaction was stopped, the absorbance at 450 nm was measured with the SpectraMax Paradigm Multi-Mode Detection Platform (Molecular-Devices Japan). The sRAGE concentration was calculated from the absorbance measurements using a calibration curve created with known concentrations (13.72-10,000 pg/mL) of recombinant mouse RAGE.

Statistical analysis

Data were expressed as mean \pm standard deviation (SD). All statistical analyses were performed using SPSS II software (IBM Japan, Tokyo, Japan). The Kruskal-Wallis approach was used for intergroup comparison. Differences were considered significant at a significance level of 5%.

Results

TNF α production in response to AGEs, glycation intermediates and AGE-modified HSA

The measured values of TNF α content in culture supernatants collected 6 hours after sample application are shown in **Fig.1**. The levels of TNF α was decreased (7.1-44.6 pg/mL) after the application of free AGEs (PEN, CML and CEL) and glycation intermediates (3DG, GO and MGO)

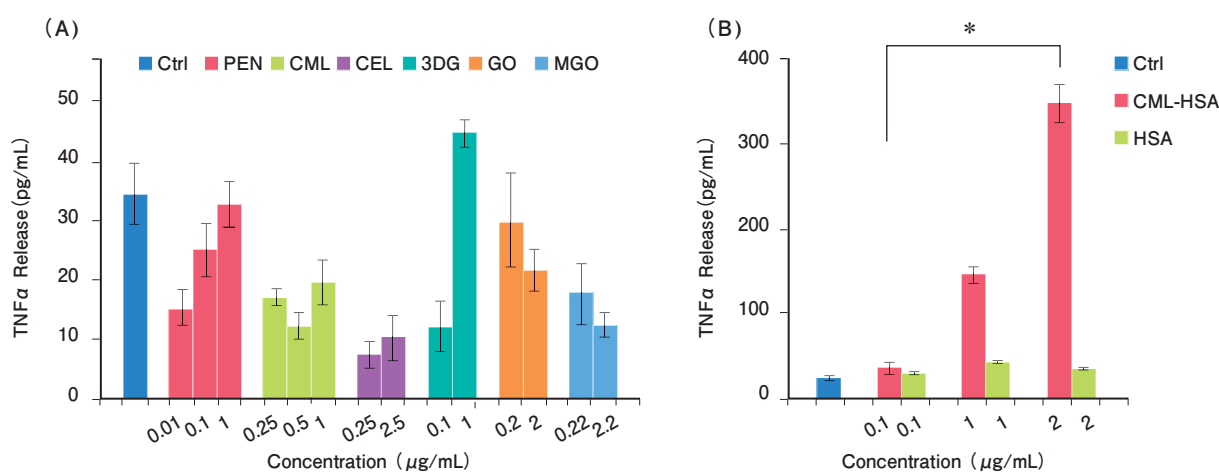


Fig.1. Effect of AGEs, glycation intermediates and AGE-modified HSA on TNF α production from RAW264.7 cells.

Each AGE or glycation intermediate was diluted in DMEM at a concentration of 0.01-2.5 μ g/mL and applied to RAW264.7 cells. Six hours after application, the culture supernatant was collected and measured for TNF α content by ELISA ($n = 3$). Detected TNF α was dose-dependently increased after the application of CML-HSA, a protein-bound AGE (B), but not with free AGEs (PEN, CML and CEL) and glycation intermediates (3DG, GO and MGO) (A). Data are expressed as mean \pm SD (* $p < 0.05$).

PEN: pentosidine, CML: *N* ϵ -carboxymethyl-L-lysine, CEL: *N* ω -carboxyethyllysine, 3DG: 3-deoxyglucosone, GO: glyoxal, MGO: methylglyoxal

(Fig.1A), whereas the application of CML-HSA, a protein-bound AGE, resulted in a dose-dependent increase in TNF α at a CML-HSA concentration of 0.1-2 μ g/mL (Fig.1B).

Time-course of TNF α production after the application of CML-HSA and LPS

The measured values of TNF α content in culture supernatants collected at 1, 3, 6, 12 and 24 hours after the sample application are shown in **Fig.2**. At all concentrations of CML-HSA, TNF α production reached the peak at 3 hours after the application of CML-HSA at 0.5, 1, 2 μ g/mL and declined thereafter. In contrast, after the application of LPS, the TNF α production did not decline even after 3 hours of incubation and it continued to increase up to 24 hours.

TNF α production in response to AGE-modified proteins

Table 2 summarizes the content of CML and fluorescent AGEs in AGE-modified proteins. The production of each component varied depending on the type of protein modified by AGEs. Increased CML production was observed in the sample of AGE-modified keratin.

Measured values of TNF α content in culture supernatants collected 3 hours after the sample application are shown in

Table 2. The content of CML and fluorescent AGEs in the AGE-modified proteins (GO2+).

Protein	CML content (μ g/mL)	Content of fluorescent AGEs (count)
HSA	0.48 \pm 0.25	76.5 \pm 0.49
Keratin (wool-derived)	4.57 \pm 0.28	68.0 \pm 0.11
Collagen-peptide (cowhide-derived)	0.34	71.9

(HSA, keratin: $n = 3$, collagen-peptide: $n = 2$)

Macrophage Response to Glycation Stress

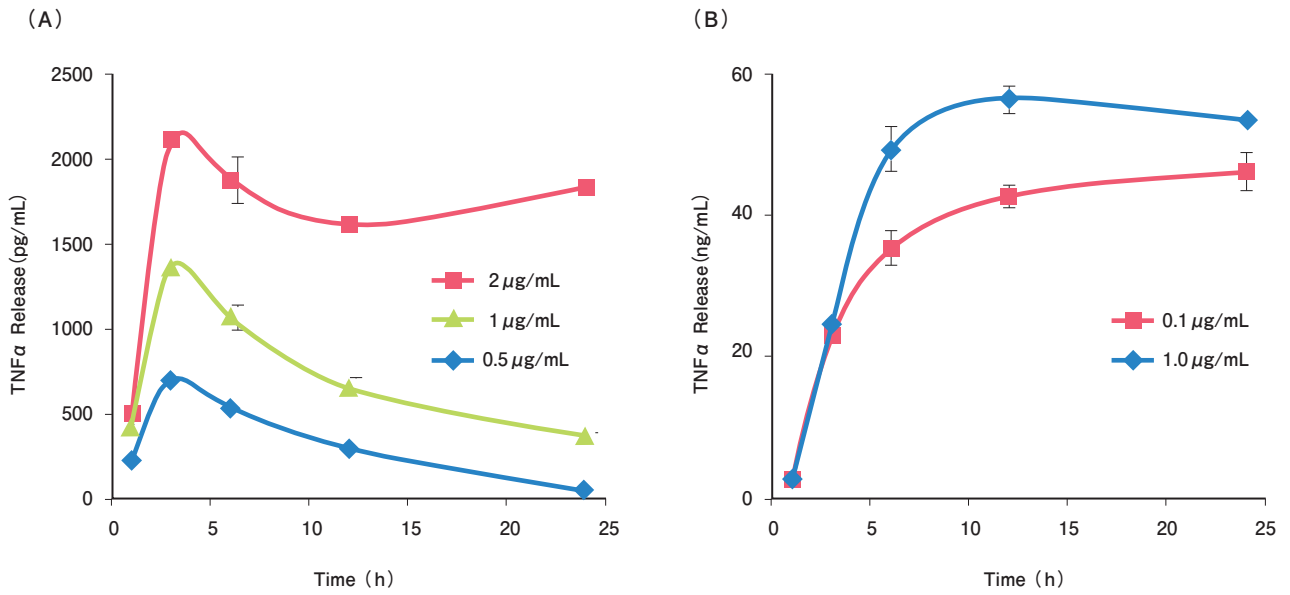


Fig.2. Time-course of TNF α production after the application of CML-HSA (A) and LPS (B).

CML-HSA diluted at a concentration of 0.5-2 $\mu\text{g}/\text{mL}$ or LPS diluted at 0.1-1 $\mu\text{g}/\text{mL}$ was applied to RAW264.7 cells. The culture supernatant was collected at 1, 3, 6, 12 and 24 hours after application and measured for TNF α content by ELISA ($n = 3$). (A) After application of CML-HSA, TNF α production reached the peak at 3 hours after application and declined thereafter. (B) In contrast, after the application of LPS, the TNF α production continued to increase even after 3 hours of incubation and it remained at high levels until 24 hours. Data are expressed as mean \pm SD.

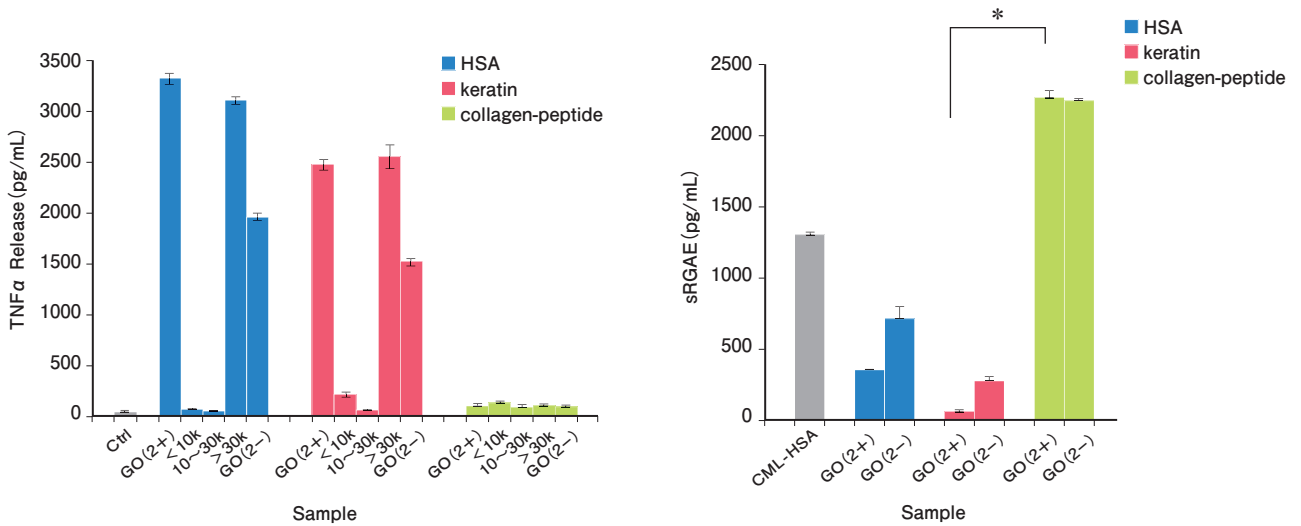


Fig.3. Effect of AGE-modified proteins on TNF α production from RAW264.7 cells.

Each of the prepared AGE-modified proteins was diluted to 1:5 in DMEM and applied to RAW264.7 cells. The culture supernatant was collected 3 hours after the application and measured for TNF α content by ELISA ($n = 3$). TNF α production increased remarkably after the application of AGE-modified HSA or keratin in the presence of GO (2+), but not with collagen-peptide. Data are expressed as mean \pm SD.

Fig.4. Effect of AGE-modified proteins on sRAGE production from RAW264.7 cells.

Each of the prepared AGE-modified proteins was diluted to 1:5 in DMEM and applied to RAW264.7 cells. The culture supernatant was collected 3 hours after the application and measured for sRAGE content by ELISA ($n = 3$). sRAGE levels increased after the application of AGE-modified collagen-peptide, but not with HSA or keratin. Data are expressed as mean \pm SD (* $p < 0.05$).

Fig.3. TNF α production was remarkably increased after the application of AGE-modified HSA or keratin, but not with collagen-peptide. No correlation was observed between the content of CML or fluorescent AGEs in each sample and the TNF α production.

The molecular weight of each protein are HSA (66,437)⁸⁾, keratin (84,000)⁹⁾, and collagen-peptide (1,000). >30K samples contain HSA, keratin, AGEs-HSA and AGEs-keratin. The difference of TNF α between >30K and GO (2-) appears to be related to production of AGEs, since GO (2-) shows the effect of the proteins themselves.

Fig.4 shows the concurrently measured values of sRAGE content. In contrast to TNF α production, sRAGE production was remarkably increased after the application of AGE-modified collagen-peptide.

Discussion

Establishment of a model for evaluating TNF α production in response to glycation stress using RAW264.7 cells

Previous studies showed the TNF α response of RAW264.7 cells to inflammation-inducing substances. The application of a peptide (AQARSAASKVKVSMKF) contained in laminin-10, a major protein component of the basement membrane, to RAW264.7 cells detected the TNF α at 3 hours after the application and it continued increase up to 24 hours¹⁰⁾. A component of beta-glucan (Zymosan A), has also been shown to induce TNF α production by RAW264.7 cells, where TNF α production is significantly increased at 7 hours after the application of zymosan A and reaches the peak at 12.5 hours¹¹⁾. However, the present findings show that CML-HSA-induced TNF α activity reached the peak at 3 hours after the application and declined immediately. This suggests that the inflammation-inducing mechanism of AGEs is different compared to previous inflammation-inducing mechanism mentioned above. The

present findings also suggest that the evaluation of AGE-induced inflammation tends to be affected by incubation time and that the inflammatory reaction occurs immediately after the production of AGEs *in vivo*.

Based on these findings, we established a model for evaluating the AGE-induced inflammatory reaction as shown in **Fig.5**.

Expression of the AGE-induced inflammatory reaction

Many cell-surface receptors or proteins recognize AGEs as ligands including RAGE, macrophage type-I and type-II class A scavenger receptors (MSR-A) and class B scavenger receptors, such as CD36 and SR-BI, lectin-like oxidized low density lipoprotein receptor-1 (LOX-1), galactin-3 complex, fasciclin, EGF-like, laminin-type EGF-like, and link domain-containing scavenger receptor-1 and 2 (FEEL1/2), megalin, and toll-like receptor 4 (TLR4)¹²⁾. Among the receptors, pattern recognition receptors (RAGE and TLR4) activate intracellular signal; other receptors involved in AGEs-uptake and breakup¹³⁾. RAGE induces an inflammatory reaction when activated by CML and other substances modified with glyceraldehyde or glycolaldehyde¹⁴⁾. Inflammation response is different among ligands: those that oligomerize activate RAGE more highly¹⁵⁾. In this study, unlike the application of collagen-peptide, AGE-modified keratin highly induced TNF α production, which may result from intracellular activation induced by keratin oligomerization with S-S bond.

A study using the rat adrenal pheochromocytoma cell line, PC12, showed an increased NF- κ B activity induced by PEN¹⁶⁾. Although PEN induced much less TNF α in this study, NF- κ B activity may be increased. No correlation was observed between the content of CML in each AGEs-protein sample and the TNF α production (Fig.3), suggesting that inflammation reaction induced by AGEs activate NF- κ B through TLR4 as well as RAGE. No information was available on these receptors and the cellular response in the cultured macrophage cell line used in this study, which is one of the limitations of the present study.

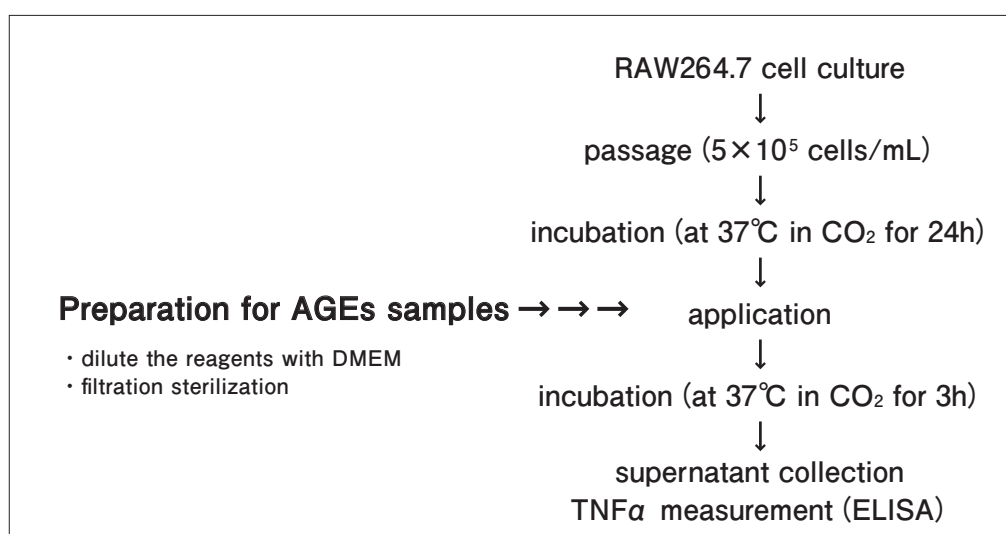


Fig.5. An experimental model for evaluating the AGE-induced inflammatory reaction.

RAW264.7 cells were subcultured at a density of 5×10^5 cells/mL. AGE samples, diluted in DMEM and sterilized by filtration, were applied to RAW264.7 cells after incubation at 37°C in CO₂ for 24 h. Three hours after incubation, the culture supernatant was collected and measured for TNF α content by ELISA.

Potential of inflammation induction by AGEs in vivo

The concentrations of different AGEs and glycation intermediates in human blood, as reported previously, are summarized in [Table 3](#)^{17)~21)}. The blood CML concentration is increased in patients with type 2 diabetes complicated by coronary artery disease ($0.530 \pm 0.084 \mu\text{g/mL}$), as compared to normal individuals ($0.408 \pm 0.077 \mu\text{g/mL}$). The concentration of CML-HSA *in vivo* is unknown, since it is not clear how much free CML combines to HSA in blood. CML-HSA ($0.5 \mu\text{g/mL}$) induced TNF α , suggesting that AGEs induce inflammatory reaction *in vivo* at a concentration of AGEs similar to that in the human body.

Table 3. The concentrations of different AGEs and glycation intermediates in human blood.

	Normal	Diabetes
PEN ¹⁷⁾	$1.63 \pm 0.07 \text{ pmol/mg}$	$2.09 \pm 0.16 \text{ pmol/mg}$
CML ¹⁸⁾	$0.408 \pm 0.077 \mu\text{g/mL}$	$0.530 \pm 0.084 \mu\text{g/mL}^*$
CEL ¹⁹⁾	$128.8 \pm 12 \text{ ng/mL}$	—
3DG ²⁰⁾	$199 \pm 53 \text{ nmol/L}$	$353 \pm 110 \text{ nmol/L}$
GO ²¹⁾	$0.05 - 0.08 \mu\text{g/mL}$	$0.19 - 0.33 \mu\text{g/mL}$
MGO ²¹⁾	$0.04 - 0.1 \mu\text{g/mL}$	$0.2 - 0.29 \mu\text{g/mL}$

* Complicated by coronary artery disease

Potential of collagen-peptide to induce sRAGE

Previous studies have demonstrated that the peptide AQARSAASKVKVSMKF, as mentioned before, induces MMP-9 production¹⁰⁾ and that plasmin, a fibrinolytic factor in blood, is involved in the transformation of proMMP, a latent form of MMP, to the active form of MMP²²⁾. Although the precise effect of collagen-peptide on MMP is unknown, it is possible that collagen peptide indirectly induces sRAGE expression by increasing MMP activity, which result in decreasing TNF α production through lack of full-length transmembrane form of RAGE.

Conclusion

We established a model for evaluating TNF α in response to glycation stress using RAW264.7 cell culture system. The results suggest that the tissue accumulation of AGEs may be responsible for the development of macrophage-mediated inflammation.

Part of this study was presented at the 13th Annual Meeting of Japanese Society of Anti-Aging Medicine (June 2013, Yokohama, Japan).

Conflict of interest

The authors have no conflict of interest in this study.

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