

Effect of advanced glycation end products, extracellular matrix metalloproteinase inducer and matrix metalloproteinases on type-I collagen metabolism

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Abstract. The aim of the study was to examine the association among advanced glycation end products (AGEs), extracellular matrix metalloproteinase inducer (EMMPRIN) and matrix metalloproteinase (MMPs), and investigate whether AGEs affect type I collagen (COL-I) through EMMPRIN or MMPs. A co-culture system with the osteoblast-like cells (MC3T3E1) and mouse RAW264.7 cells was employed to examine the effects of AGE-bovine serum albumin (BSA) (50 mg/l), EMMPRIN antibody (5 mg/l) and AGE-BSA+EMMPRIN antibody separately on COL-I expression for 24 h. Culture media were analyzed for the content of COL-I by ELISA. The effect of different concentrations of AGE-BSA (0, 50, 100, 200 and 400 mg/l) for 24 h was assessed on COL-I levels. Finally, semiquantitative RT-PCR was used to detect the osteoblast COL-I mRNA expression and MMP-2 and MMP-9's PMAO were also measured in the culture medium. COL-I content in the culture medium decreased significantly following treatment with AGE-BSA ($P<0.05$). EMMPRIN antibody increased COL-I content ($P<0.05$). EMMPRIN antibody+AGE-BSA increased COL-I significantly ($P<0.05$). Different concentrations of AGE-BSA increased COL-I mRNA expression significantly compared with the control group ($P<0.05$), and were enhanced with increasing AGE-BSA concentration ($P<0.05$). Also MMP-2 and MMP-9 secretion increased significantly ($P<0.05$), with the increasing AGE-BSA concentration. In conclusion, an increase in AGE levels *in vitro* stimulates the secretion of EMMPRIN/MMPs, promotes the degradation of COL-I and reduces bone strength.

Introduction

Type I collagen (COL-I) is the main component of bone matrix and its main function is to absorb and transfer stress and is suitable for force deformation and tension maintenance (1). Matrix metalloproteinase (MMPs) is a major degradation enzyme system of extracellular matrix that can degrade extracellular matrix components such as collagen and elastin, but not polysaccharides (2). Synthesis and expression of MMPs are regulated by the extracellular matrix metalloproteinase inducer (EMMPRIN).

Previous findings (3,4) have shown that advanced glycation end products (AGEs) may not support bone resorption to some extent, but reduces bone resorption and leads to osteoporosis. Additionally, patients with osteoporosis have a lot of AGEs accumulated in their tissues. The accumulation can decrease the mechanical properties of cortical bone and trabecular bone and increase osteopsathyrosis, and increases the risk of fractures (5).

In the present study, we examined the effect of the AGE-bovine serum albumin (BSA) *in vitro* on the osteoblast and osteoclast precursor cell co-culture system, and observed that COL-I expression is controlled by AGEs and the EMMPRIN/MMPs system.

Materials and methods

Cells and reagents. The American ABI, type 9700 polymerase chain reaction (PCR) machine was used in the present study [Applied Biosystems (ABI) Life Technologies, Foster City, CA, USA]. BSA was purchased from Amresco LLC (Solon, OH, USA); α -minimum essential medium (α -MEM) from Gibco (Grand Island, NY, USA) and fetal bovine serum from Hangzhou Sijiqing Biology Engineering Materials Co., Ltd. (Hangzhou, China). Mouse COL-I ELISA kit was purchased from the American Research and Experimental Development Corporation. MMP-2 and MMP-9 reagents, and EMMPRIN antibody were purchased from Shanghai Senxiong Science and Technology Industrial Co., Ltd. (sc-25531; Shanghai, China). Mouse bone-forming cells (MC3T3E1) and mouse macrophage RAW264.7 cells were purchased from CAS Shanghai Life Science Institute (Shanghai, China).

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Table I. Effect of different AGE-BSA concentrations in a co-culture system on the expression of MMP-2 and MMP-9 and COL-I mRNA.

Items	α -MEM	BSA	AGE-BSA					
			50	100	200	400	Osteoblast 400	Osteoclast 400
COL-I/ β -actin mRNA	0.7 \pm 0.02	0.7 \pm 0.02	0.8 \pm 0.02 ^{a,b}	0.9 \pm 0.03 ^{a,b}	0.9 \pm 0.03 ^{a,b}	1.4 \pm 0.05 ^{a,b}	1.1 \pm 0.05 ^{a,b}	-
MMP-2 (INT x mm ²)	41.5 \pm 3	40.9 \pm 3	75.3 \pm 5 ^{a,b}	117.6 \pm 10 ^{a,b}	138.7 \pm 13 ^{a,b}	158 \pm 15 ^{a,b}	41.0 \pm 3 ^{a,b}	38.9 \pm 3 ^{a,b}
MMP-9 (INT x mm ²)	211 \pm 19	144 \pm 15	451.3 \pm 30 ^{a,b}	578.9 \pm 39 ^{a,b}	667.0 \pm 49 ^{a,b}	751 \pm 55 ^{a,b}	100.2 \pm 9 ^{a,b}	72.1 \pm 5 ^{a,b}

^aP<0.05, compared with the α -MEM group; ^bP<0.05, compared with BSA group. MMP, matrix metalloproteinase; AGE, advanced glycation end product; BSA, bovine serum albumin; α -MEM, α -minimum essential medium.

AGE-BSA preparation. The concentration of BSA was 5 g/l, and that of glucose was 50 mmol/l, in sterile phosphate-buffered saline (PBS at pH 7.4). Solutions were kept at 37°C under sterile conditions and in the dark for 90 days. Unreacted glucose in PBS solution was removed by dialysis and the formed AGE-BSA was collected. Fluorescence spectrum scanning analysis with an excitation wavelength of 370 nm, an emission wavelength of 440 nm, and a slit of 3 nm, was used for identification of the AGE. The glucose that did not contain BSA served as the negative control.

Cell culture and intervention. MC3T3E1 and RAW264.7 cells were cultured in 10% fetal bovine serum and α -MEM at 37°C in a 5% CO₂ incubator. The cells were grown in logarithmic phase in 6-well plates and the RAW264.7 cells were transferred into transwells. When the cells grew to 80-90% confluency, the RAW264.7 cells were inoculated from the transwell to the well with MC3T3E1 cells. The cell growth was then synchronized by incubation in serum-free culture medium (starvation conditions) for 24 h.

The co-cultured cells were treated using 50 mg/l AGE-BSA, 5 mg/l EMMPRIN antibody, and combined AGE-BSA and EMMPRIN antibody treatments, respectively, for 24 h. The controls were treated with BSA (400 mg/l), for 24 h and the culture medium was collected to detect the levels of COL-I.

The cells were treated with different concentrations of AGE-BSA (0, 50, 100, 200 and 400 mg/l) in a co-culture system with BSA (400 mg/l) as the control to examine the expression of COL-I. The cells and culture medium were collected after 24 h to detect the COL-I level and the secretion of MMP-2 and MMP-9.

Detection of COL-I in the culture medium by ELISA. Release of COL-I in the medium was measured using ELISA, as previously described (2).

Detection of osteoblast COL-I mRNA expression by RT-PCR. Following the treatments, total RNA of the cells was extracted using TRIzol one-step method. Total RNA (2 μ l) was used for reverse transcription, employing primers for COL-I upstream, 5'-TCTCCACTCTTCTAGTTCCT-3', and downstream,

5'-TTGGGTCATTTCCACATGC-3' to amplify a 268 base pair (bp) length cDNA fragment. Primers used for β -actin were upstream, 5'-CGTGCCTGACATTAAAGAG-3', and downstream, 5'-CTGGAAGGTGGACAGTGAG-3', to amplify a 435 bp length DNA fragment. The RT-PCR condition used were: 94°C denaturation for 3 min, and again at 94°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 1 min for 30 cycles of amplification, followed by extension at 72°C for 10 min.

Gelatin zymography to detect MMP-2 and MMP-9 in the culture medium. Cell culture medium was processed for SDS-polyacrylamide gel electrophoresis and at low temperature (4°C) for 2.5 h. The gel was placed in 2.5% Triton X-100 and washed for 1 h and the gel was soaked in incubation buffer (50 mmol/l Tris-HCl, 5 mM CaCl₂, 0.2 mmol/l NaCl, 0.02% Brij-35, pH 7.6; Shanghai Zhenxing Reagent Factory, Shanghai, China) at 37°C for 18 h. The gel was then stained with coomassie brilliant blue and finally the MMP bands were visualized on a blue background and analyzed densitometrically.

Statistical analysis. SPSS 16.0 statistical software (SPSS, Inc., Chicago, IL, USA) was used to analyze data, and a comparison between groups was made using the Student-Newman-Keuls test. P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of AGE-BSA and EMMPRIN antibody. The effect of AGE-BSA and EMMPRIN antibody, as well as combined AGE-BSA and EMMPRIN antibody on COL-I content in a co-culture system is shown in Table I. Under basic conditions, COL-I secretion was observed in the culture medium. In the presence of 50 mg/l AGE-BSA for 24 h, the content of COL-I decreased significantly. By contrast, the addition of antibody of EMMPRIN resulted in COL-I content increasing significantly, even in the presence of AGE-BSA (P<0.05).

Effect of different concentrations of AGE-BSA in the co-culture system on MMP-2 and MMP-9 secretion and COL-I mRNA

expression. The addition of BSA had no effect on the relative expression of COL-I mRNA compared with the α -MEM group ($P>0.05$). AGE-BSA at different concentrations (50, 100, 200 and 400 mg/l) increased the relative expression of COL-I significantly compared with the BSA and α -MEM groups ($P<0.05$), and increased proportionally with the increase in the AGE-BSA concentration ($P<0.05$). Secretion of MMP-2 and MMP-9 increased significantly ($P<0.05$), and increased proportionally with the increase in the AGE-BSA concentration ($P<0.05$) (Table I).

Discussion

COL-I is the most abundant structural protein in bone, accounting for 90% of organic matter, is a bone metabolic product and has the ability of adjusting bone metabolism. Procollagen type I carboxy-terminal propeptide (PICP) and COL-I procollagen type I amino-terminal propeptide (PINP) constitute the specific index of COL-I synthesis rate, although a daily rhythmicity (6) between PICP and PINP is present that should be taken into account when assessing COL-I levels. COL-I is easy to undergo non-enzymatic glycosylation and osteoporosis patients have a large amount of AGEs accumulated in their bodies, which can decrease the collagen content and cause BMD reduction and bone biomechanical changes, leading to osteoporosis. Thus AGEs are associated with the synthesis and degradation of COL-I (7).

The findings of the present study showed that in the co-culture system MC3T3E1 expressed COL-I in the α -MEM group. With the addition of AGE-BSA, COL-I gene expression increased in a concentration-dependent manner. At the same time, COL-I content in the culture medium of cells decreased with the increase in AGE-BSA concentration. These results are similar to those of the reports by Yamamoto and Ozono (7) and indicate that although the osteoblast synthesis of COL-I increased in the co-culture system, the total number of cells *in vitro* decreased. Previous studies (8) have shown that AGEs can increase the expression of EMMPRIN by MC3T3E1 cells and elevate the degradation of extracellular matrix.

MMPs are a class of proteases that hydrolyze many types of proteins in extracellular matrix in the presence of zinc ions. There are a variety of bone MMPs, of which MMP-2 was mainly formed by osteoblasts and secreted, while MMP-9 is mainly secreted by osteoclasts. The present study identified that MMP-2 and MMP-9 levels increased after the AGE-BSA addition, in a dose-dependent manner. Previous studies (9) also showed that the upstream products of MMP-2 and MMP-9 were increased by AGEs. When EMMPRIN was constrained, the content of MMP-2 and MMP-9 decreased significantly (10).

The findings of the present study show that antibody against EMMPRIN antagonizes MMP-2 and MMP-9, with increased content of COL-I in the culture medium. This result indicates that AGEs increase the secretion of MMP-2 and MMP-9 by promoting EMMPRIN expression, thereby promoting the degradation of COL-I.

In summary, AGEs can promote the *in vitro* secretion of MMP-2 and MMP-9, enhancing COL-I degradation. Although COL-I gene expression showed an increase, its degradation may be more than the synthesis. This tilts the balance between COL-I synthesis and degradation, and affects the metabolism of the bone matrix, reduces bone strength and causes pathogenesis of osteoporosis (11).

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