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In situ accumulation of advanced glycation endproducts (AGEs) in bone matrix and its correlation with osteoclastic bone resorption

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ABSTRACT

Advanced glycation end products (AGEs) have been observed to accumulate in bone with increasing age and may impose effects on bone resorption activities. However, the underlying mechanism of AGEs accumulation in bone is still poorly understood. In this study, human cortical bone specimens from young (31 ± 6 years old), middle-aged (51 ± 3 years old) and elderly (76 ± 4 years old) groups were examined to determine the spatial–temporal distribution of AGEs in bone matrix and its effect on bone resorption activities by directly culturing osteoclastic cells on bone slices. The results of this study indicated that the fluorescence intensity (excitation wave length 360 nm and emission wave length 470 ± 40 nm) could be used to estimate the relative distribution of AGEs in bone (pentosidine as its marker) under an epifluorescence microscope. Using the fluorescence intensity as the relative measure of AGEs concentration, it was found that the concentration of AGEs varied with biological tissue ages, showing the greatest amount in the interstitial tissue, followed by the old osteons, and the least amount in newly formed osteons. In addition, AGEs accumulation was found to be dependent on donor ages, suggesting that the younger the donor the less AGEs were accumulated in the tissue. Most interestingly, AGEs accumulation appeared to initiate from the region of cement lines, and spread diffusively to the other parts as the tissue aged. Finally, it was observed that the bone resorption activities of osteoclasts were positively correlated with the *in situ* concentration of AGEs and such an effect was enhanced with increasing donor age. These findings may help elucidate the mechanism of AGEs accumulation in bone and its association with bone remodeling process.

Introduction

Advanced glycation end products (AGEs) are a chemical modification of long-lived proteins by sugars [1,2]. Initially, a covalent bond is formed between reducing sugars (*e.g.*, glucose) and free amino groups (e.g., lysine and arginine residues) in the proteins [3]. Subsequent reactions cause an irreversible, non-enzymatic post-translational modification of proteins (*i.e.*, the Maillard reaction) and give rise to the formation of glucose-mediated intermolecular cross-links [4]. Long-lived proteins, such as collagen, are vulnerable to such non-enzymatic modification over time. Among the heterogeneous class of AGEs structures in bone, pentosidine is an intermolecular cross-link and a fluorescent component formed *in vivo* between collagen molecules, which has been chemically well-described [4]. It can also be produced *in vitro* by non-enzymatic reaction of ribose with lysine and arginine residues [5].

AGEs may impose detrimental effects on bone remodeling: a coupling process of bone resorption by osteoclastic cells and bone formation by osteoblastic cells. *In vitro* studies from osteoblastic cell cultures have shown that AGE-modified collagen was able to inhibit the proliferation and differentiation of osteoblastic cells [6–9]. Additionally, the introduction of AGE-modified bovine serum albumin to cultures of human osteoblast-like cells resulted in a significantly reduced synthesis of collagen I and osteocalcin [10]. The detrimental effect of AGEs on the functional alteration of osteoblasts may involve apoptosis of osteoblastic cells [11,12] and various molecular pathways. These pathways include autocrine–paracrine pathway such as IGF-I and its binding proteins [8], oxidative stress pathway [7], cytokine pathway such as Interleukin-6, and growth factors pathway such as TGF-β type II receptor [6]. Although a number of studies have investigated the influence of AGEs on osteoblastic cells and bone formation, controversial results were reported regarding the effect of AGEs on osteoclast activities. Using mouse bone cell cultures and an *in vivo* model of rats with subcutaneously implanted particles, some

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studies reported that AGEs may enhance osteoclast-induced bone resorption after 4 days in culture [13]. On the other hand, however, other studies on bovine bone samples indicated that AGEs may inhibit the resorption process after matrix pentosidine was introduced in osteoclasts cell culture for 8 days [14].

Although numerous studies have shown that matrix AGEs may alter the ultrastructure of bone matrix and affect bone remodeling process, little information is available in the literature on the mechanism of the spatial and temporal accumulation of AGEs in bone and its effect on bone remodeling process. To this end, the objectives of this study were: 1) to confirm the efficacy of using epifluorescence intensity for assessing the relative *in situ* concentration of AGEs in bone; and 2) to examine the spatial and temporal distributions of AGEs in human cortical bone; and 3) to scrutinize the correlation of *in vitro* osteoclast activities in bone resorption with the accumulation of AGEs.

Materials and methods

Specimen preparation

Cortical bone samples were harvested from eighteen (18) cadaveric femurs of male human donors from young (N = 6, 31 ± 6 years old), middle-aged (N = 6, 51 ± 3 years old) and elderly (N = 6, 76 ± 4 years old) age groups. The femurs were acquired from the National Disease Research Interchange (Philadelphia, PA) and screened for known bone diseases. First, a slab of cortical bone was cut from the middle shaft of each femur using a band saw. Next, cross sectional slices with a dimension of 3 mm × 3 mm × 0.3 mm were sectioned from the medial quadrant of the slab using a low feeding speed diamond saw (Isomet 2000, Buehler, Lake Bluff, IL). Finally, these bone slices were ground and polished to a thickness of 0.15 mm with successive grits of sand papers until microstructure features (*e.g.*, osteons) were clearly observed through an optical microscope.

Decalcification

To ensure consistent results, a pilot study was conducted to determine the time period that is required to completely remove the mineral phase by immersing bone slices in the formic acid solution [15] with the assistance of a shaker at room temperature. In order to inhibit bacterial contamination, 0.01% sodium azide was added in the solution. Four bone slices (3 mm × 3 mm × 0.15 mm) from a femur were decalcified for 2, 4, 8 and 16 days, respectively, to monitor the progress of decalcification. The completion of decalcification was verified by checking the residual calcium content in the specimen using energy dispersive spectroscopy (EDS). Briefly, after decalcification, the specimens were gradually dehydrated by soaking in 50%, 60%, 70%, 80%, 90%, and 100% alcohol solutions for 1 h each with the solutions being changed every 20 min. After dehydration, the specimens were coated with carbon by sputter deposition to provide electrical conductivity for scanning electron microscopy (SEM) imaging. All the specimens were imaged using a scanning electronic microscope (Jeol SEM-850, Tokyo, Japan) at an accelerating voltage of 20 kV and a working distance of 15 mm. The elemental composition was measured by electron probe microanalysis using energy dispersive spectroscopy (EDS) (INCA x-sight model 7636, Oxford Instruments America, Concord, MA). For each specimen, the percent concentration of calcium at cement lines, osteons, and interstitial regions was inspected using EDS to verify the completeness of decalcification. The results indicated that the bone slices could be decalcified completely in 8 days (Table 1) for all different regions (*i.e.*, osteons, cement lines, and interstitial tissue). Based on the result of this pilot study, 8-day was finally selected for demineralization of bone specimens.

Table 1
Calcium presence in decalcified bone samples (EDS wt.% calcium).

	2 Days	4 Days	8 Days	16 Days
Cement line	0.00	0.00	0.00	0.00
Osteons	0.06	0.00	0.00	0.00
Interstitial bone	0.07	0.03	0.00	0.00

Fluorescence microscopy

The fluorescence distribution in the bone slices was examined using an epifluorescence microscope (Leica DM5500B, Leica Microsystems Inc., Bannocoburn, IL) with an excitation filter of 360 nm and a barrier filter of 470 ± 40 nm. First, the bone slices were mounted on a regular microscopic slide with a cover slip. Then, the fluorescence and bright-field images of the bone slices were taken using a high-resolution video camera (Fig. 1) under the same exposure time (100 ms) to ensure a consistent condition of data acquisition. For each bone slice, the fluorescent intensity was assessed at three different anatomic sites: *i.e.*, the newly formed osteons (referred to as new osteons), the osteons formed in previous bone remodeling cycles (referred to as old osteons), and the remnant of the tissue (referred to as interstitial tissue) (Fig. 1b). The average fluorescence intensity of each tissue type was quantified using a public domain Image J program (developed at the U.S. National Institutes of Health).

Quantifying pentosidine content in bone using HPLC

Correlation between fluorescence intensity and pentosidine content was verified using the following procedure: First, six fields of view were randomly chosen for each bone slice. A group (N = 6) of new osteons, old osteons, and interstitial tissues was identified from each field of view (100×) of the epifluorescence microscope, respectively (Fig. 2a). The fluorescence intensity of each tissue type was then calculated as the average value of the pooled samples from the six fields of view. Second, the new osteons, old osteons, and interstitial tissues chosen for the fluorescence intensity measurement (Fig. 2b) were dissected using a pair of tweezers with ultrafine tips (Dumont#5, World Precision Instruments, Sarasota, FL) under a dissection microscope (415 TBL-10, National Optical, San Antonio, TX) and pooled together to each tissue type. Third, the concentration of pentosidine for each tissue type was measured following a well established HPLC protocol reported in our previous studies [16,17]. Briefly, the dissected bone tissues were hydrolyzed in a polyethylene microcentrifuge vial, containing 500 μl of 6 M HCl, at 110 °C for 24 h. The acid was removed by drying in a SpeedVac centrifuge (SPD111V, Thermo Savant, Holbrook, NY) at 60 °C. The residues were then dissolved in 200 μl of ultra-pure water containing pyridoxine as internal standard. Three quarters of the re-suspended residue (150 μl) were filtered, diluted and injected into a HPLC system (System Gold 126 Solvent Module, Beckman Coulter, Fullerton, CA). One quarter of the re-suspended residue (50 μl) was utilized to measure collagen content by an established colorimetric method [18–20]. The concentration of pentosidine in each sample was then measured and normalized by the total amount of collagen as millimoles per mole collagen (mmol/mol).

Immunohistochemistry

Spatial distribution of AGEs in human cortical bone was further confirmed by immunofluorescence with anti pentosidine mouse monoclonal antibody (Clone No. Pen-12, Cosmo Bio, Tokyo, Japan). The anti pentosidine antibody was purified by protein G affinity chromatography from the cell cline (PEN-12), which was grown in ascitic fluid of BALB/c mouse immunized with pentosidine-HSA. The anti pentosidine mouse antibody has high sensitivity for detection of