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PERSPECTIVES

Advanced Glycation End-products and Bone Fractures

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Abstract

Bone does not turn over uniformly, and becomes susceptible to post-translational modification by non-enzymatic glycation (NEG). NEG of bone causes the formation of advanced glycation end-products (AGEs) and this process is accelerated with aging, diabetes and antiresorptive postmenopausal osteoporosis therapy. Due to the elevated incidence of fracture associated with aging and diabetes, several studies have attempted to measure and evaluate AGEs as biomarkers for fracture risk. Here current methods of estimating AGEs in bone by liquid chromatography and fluorometric assay are summarized and the relationships between AGEs and fracture properties at whole bone, apparent tissue and matrix levels are discussed. *IBMS BoneKEy*. 2009 August;6(8):268-278.

Keywords: Advanced glycation end-products (AGEs); Bone; Mechanical properties; Non-enzymatic glycation; Pentosidine; Toughness

Introduction

Post-translational modification of proteins by the process of non-enzymatic glycation (NEG) occurs in tissues with limited turnover such as cartilage and tendon (1-2). The slow turnover process exposes matrix proteins to the extracellular environment for extended times, leading to modifications by NEG. NEG of tissues causes the formation and accumulation of advanced glycation end-products (AGEs) with age and disease, negatively impacting biomechanical properties (3-5).

Bone, in contrast, is considered to be a tissue with substantial turnover. Bone turnover rates of 5-25% per year have been reported in menopausal and postmenopausal women (6). Consequently, NEG was considered to occur in non-significant proportions and to be largely irrelevant for bone (7) except in diabetes where altered sugar metabolism caused the accumulation of AGEs (8). However, over the last decade additional evidence has emerged showing bone turnover to be a highly selective and a heterogeneous process. For example, the proportion of interstitial tissue in both cortical and cancellous bone compartments increases with chronological age and new bone formation, in the form of osteonal refilling and formation of trabecular packets, decreases with age (9-10). Also, some fragments of circumferential lamellar bone, formed during bone growth, remain unremodeled and survive well into old age (11). Thus, bone does not turn over uniformly and certain areas, analogous to tissues with slow turnover, become susceptible to post-translational modification by NEG.

In vitro cell culture studies show that NEG-modified tissue becomes more resistant to osteoclastic bone resorption (12) and causes a decrease in osteoblast proliferation and differentiation (13). Concomitant decreases in resorption and formation will decrease bone turnover, making NEG a widespread mechanism of protein modification in bone.

Several recent studies have analyzed NEG of bone under a variety of conditions in order to explain the increased fracture risk associated with aging and diabetes. In addition, since bisphosphonate therapy for postmenopausal osteoporosis slows down bone turnover, the connection between bisphosphonate therapy and NEG has also received significant attention. This *Perspective* will examine NEG of bone due to aging, diabetes and bisphosphonate treatment, with particular emphasis on AGEs and their influence on bone fracture.

Non-enzymatic Glycation (NEG) of Bone

NEG has been shown to modify both collagenous and non-collagenous matrix proteins in bone (14-17). However, due to the abundance of type I collagen in bone and its demonstrated role in bone fracture (18), AGEs in bone are reported in terms of collagen. Type I collagen in bone consists of tropocollagen molecules that contain three polypeptide chains. Each chain is a left-hand helix characterized by a unique amino acid sequence involving glycine-proline-X or glycine-X-hydroxyproline, where X is another amino acid (*e.g.*, lysine or arginine). The unique amino acid sequence makes it possible for the three polypeptide chains to wrap around each other in a right-hand sense and form a triple helix with glycine sitting at the center. The other amino acids are present at the triple helix surface. The amino acids present on the triple helix surface and at the N- and C-telopeptide terminals participate in NEG to form covalent crosslinks with their neighboring tropocollagen molecules.

NEG-mediated crosslinking involves a reaction between an aldehyde of the open chain form of glucose and the e-amino group of lysine or hydroxylysine on collagen. The resultant aldimine (glucosyl-lysine) undergoes a rearrangement to form Schiff base adduct and/or the Amadori product (19). Both Schiff base adduct and Amadori product undergo further reactions with other amino groups to form AGEs.

Advanced Glycation End-products (AGEs)

AGEs represent several intermolecular crosslinks that are formed as a result of NEG (20). To date a number of intermolecular crosslinks have been identified or proposed to occur in AGEs. These include pentosidine (21), vesperlysines (22), imidazolium compounds – methylimidazolium (MOLD (23)) and glyoxalimidazolium (GOLD (24)) – crossline (25), carboxymethyl- and carboxyethyllysine (CML and CEL (26)) and NFC-1 (non-fluorescent component-1) (27).

Out of the AGE crosslinks listed above only one, pentosidine, has been quantified in bone (15-17). However, because pentosidine is present at a low concentration of one crosslink per 200-300 collagen molecule in AGEs and bone (7;28), the estimation/measurement of bone's total AGE content is somewhat of a challenge. Current methods are discussed below.

Measurement of AGEs in Bone

Two methods for estimating AGEs in bone have been used. Both of these methods are based on the fact that a majority of the crosslinks in AGEs are fluorescent. Furthermore, controlled *in vitro* NEG reactions (29) and naturally aged proteins including lens crystallins and collagenous connective tissue (30) show classical browning associated with an increase in fluorescence.

Based on the pioneering work of Monnier and coworkers, who isolated, purified, synthesized and elucidated the structure of pentosidine (21), the first method utilizes a single-column high-performance liquid chromatography (HPLC) for the quantification of pentosidine. Under this scheme, pentosidine is separated from enzymatic crosslinks (pyridinoline (PYD) and deoxypiridinoline (DPD)) in acid-hydrolyzed bone samples in a C18 column and quantified using fluorescence (335 nm excitation; 385 nm emission) and internal standards (16;31) (Fig. 1). The amount of pentosidine, estimated from the

chromatogram, is normalized by the collagen amount present in the sample. The amount of collagen contained in each sample is approximated from its hydroxyproline content using an HPLC kit that was originally developed to estimate the amount of collagen in urine (Biorad, Germany). This method is expensive but provides rapid and precise measurement of pentosidine (the lowest detection limit is 0.02 pmol (31)). In addition, enzymatic crosslinks (PYD and DPD), providing an index of tissue maturity, can also be measured from the same HPLC run. Using this method, pentosidine can also be measured from the patient's urine and serum and used as a biomarker in a clinical setting (32-33). Because pentosidine content in bone is minimal and represents a small fraction (<1%) of total AGEs (28), the utility of pentosidine as a reliable indicator of AGE content in bone needs further evaluation.

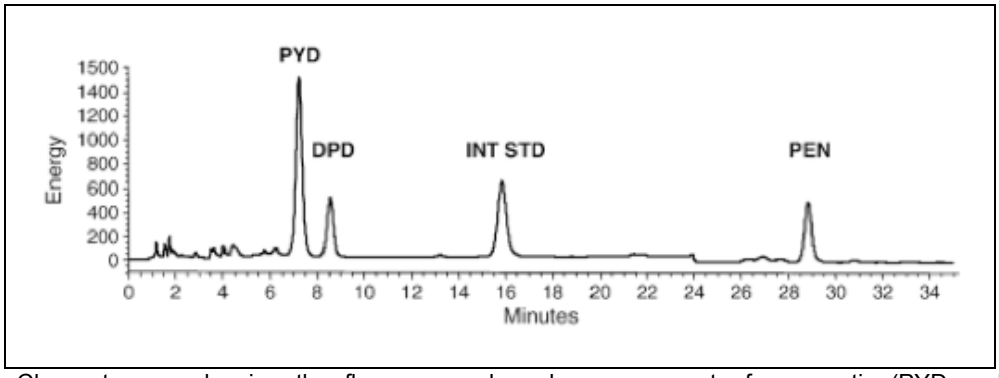

Fig. 1. Chromatogram showing the fluorescence-based measurement of enzymatic (PYD and DPD; excitation/emission: 297/395 nm) and non-enzymatic (PEN; excitation/emission: 335/385 nm) crosslinks from a 54-year-old male donor (human vertebrae L3). With permission from Elsevier (45).

The second method of measuring AGEs is based on *in vitro* NEG reactions wherein the bulk fluorescence of glycated bone is observed to increase with the progressive browning of the tissue (34) (Fig. 2). This method is also used to measure AGEs in cartilage (5). Under this scheme, demineralized bone is subjected to papain digestion that preserves acid-stable as well as acid-soluble AGE crosslinks (34-36). AGE content is then determined from the digested sample using a microplate reader (370 nm excitation and 440 nm emission) and normalized to a quinine sulphate standard. The amount of collagen in the sample is estimated based on the amount of hydroxyproline that is also measured on the microplate reader against a hydroxyproline standard at a wavelength of 570 nm (21). AGE content is then expressed as ng of quinine sulphate fluorescence/mg of collagen.

Unlike the HPLC method, the fluorometric assay is inexpensive and relatively simple to execute. More importantly, both the excitation and emission wavelengths of six different fluorescent crosslinks including vesperlysines (A, B and C), CML, CEL, PEN and crossline (21-25) are fully or partially captured by the excitation/emission wavelength of the fluorometric assay (Fig. 3). Thus it is likely that the fluorometric assay gives a more comprehensive assessment of AGE content of bone compared to HPLC-based measurement of pentosidine alone. However, unlike the HPLC method, the fluorometric assay does not precisely identify collagen as the source of measured fluorescence and may contain contributions from non-collagenous matrix proteins and cell lysates. Given that collagen represents 90% of the organic matrix in bone and there are no reports of AGEs in the cells of bone matrix (predominantly osteocytes), this limitation is likely to be minor but requires further investigation.

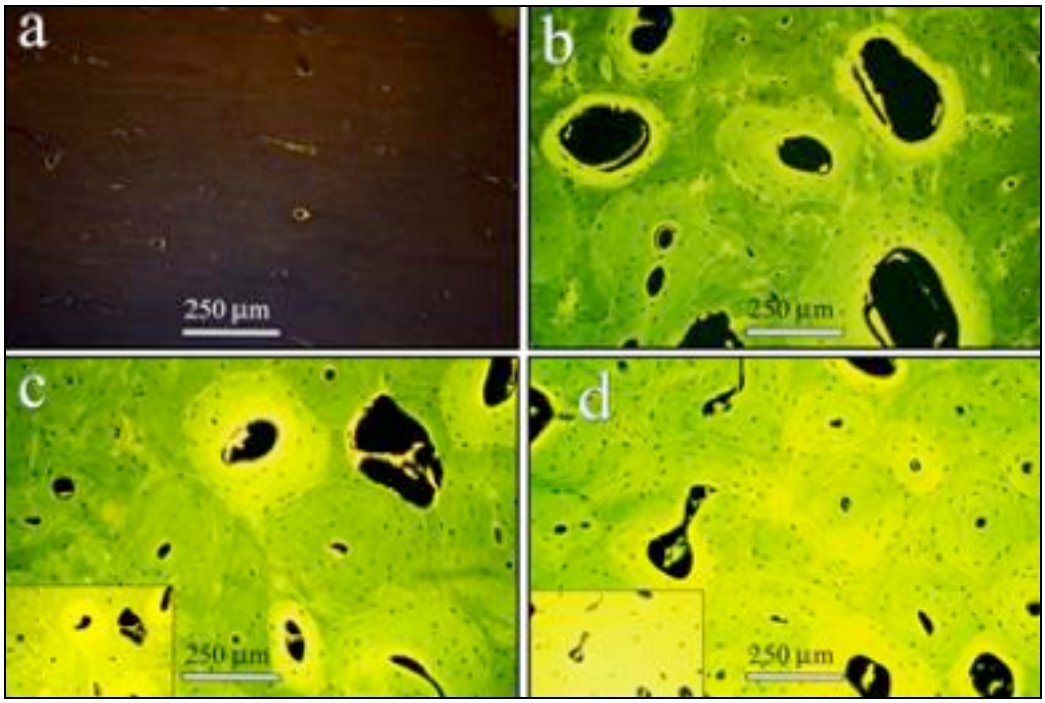

Fig. 2. Sections of *in vitro* glycated bone tissue showing a progressive increase in tissue fluorescence (excitation/emission: 370 nm/440 nm) with time of incubation (a: control; b: 3 days; c: 11 days; d: 38 days). Micrographs a–d, taken at auto-exposure, show that the increased incubation period leads to a more homogeneous glycation of cortical bone microstructure. For noting the increase in the level of fluorescence, indicative of AGE accumulation, compare to micrograph b and the insets in micrographs c and d. For the insets, the exposure was set at a fixed value (based on auto-exposure for section incubated for 3 days), and micrographs of the sections incubated for 11 and 38 days were taken at that fixed exposure. With permission from Elsevier (34).

Because fluorescence imaging is commonly used for bone, images collected at wavelengths corresponding to the fluorometric assay can also be used to provide a tissue map of the relative AGE content of bone compartments. In pioneering work Gibson *et al*. (37) applied this technique to show that, consistent with the expected tissue age in bone, AGE content was highest in circumferential lamellar bone followed by interstitial and osteonal bone. An example of cortical bone's AGE map created by this technique is shown in Fig. 4.

Bone Fracture Properties

In order to understand the relationship between AGEs and bone fracture, it is important to note a few fundamental concepts relevant to AGEs and bone biomechanics.

First, because NEG modifies only the proteins, AGEs accumulate in the organic matrix of bone. The organic matrix of bone contributes predominantly to the plastic or post-yield properties of bone. In a classic study, Burstein *et al*. (38) demonstrated that the removal of organic matrix did not affect the elastic properties of bone but completely eliminated the post-yield deformation – a major source of energy dissipation and fracture resistance in bone (39). Consistent with this finding, several studies using *in vitro* glycated bone and its matched control have demonstrated that accumulation of AGEs in bone reduces the post-yield properties of bone without altering the elastic properties (34-35;40). Thus the contributions of AGEs to bone fracture should be evaluated from post-yield properties.

Second, there are inherent differences between the two most commonly reported

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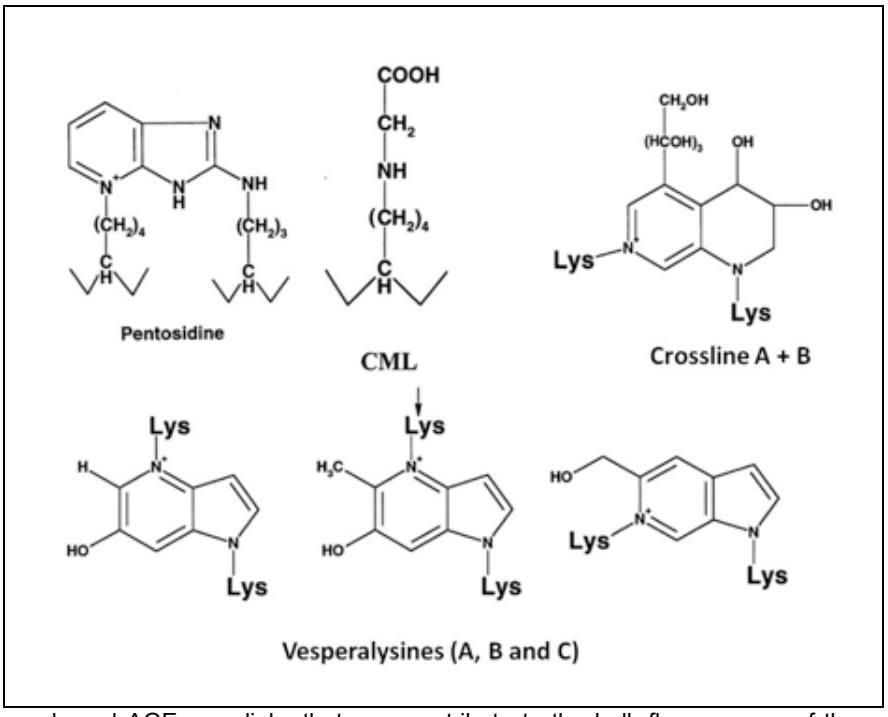

Fig. 3. Collagen-based AGE crosslinks that may contribute to the bulk fluorescence of the organic matrix that is measured using the fluorometric assay (excitation/emission: 370/440 nm). Excitation/emission wavelengths for the crosslinks shown are: pentosidine (335/385 nm), CEL/CML (340/455 nm), crossline (379/463 nm), vesperlysines A and B (366/442 nm) and vesperlysine C (345/405 nm). With permission from Elsevier (20).

bone fracture properties, strength and toughness (41). Strength (measured as maximum force normalized by bone area) represents the maximum load-carrying capacity of bone while toughness (measured as area under the load-deformation or stress-strain curve) represents a combination of the maximum load-carrying capacity as well as the maximum deformation before fracture. Because fracture in bone is strain-controlled (42) and AGEs affect the organic matrix (a known contributor to post-yield deformation), the inclusion of deformation with load-carrying capacity produces a more relevant parameter of bone fracture property. An example to support this concept is shown in Fig. 5 where the strength of *in vitro* glycated cancellous bone is higher than its matched control but the toughness is lower.

Third, bone fracture tests are generally reported at whole bone, apparent tissue and material (ECM) levels. Testing at these three levels provides variables that determine the propensity of bone to fracture. At the whole bone or organ level, the femur, radius or vertebra is subjected to non-cyclic loading until fracture and the resulting load and deformation values, representative of the bone's structural and material characteristics, are used to provide an estimate of strength or toughness (43). In the context of AGEs, whole bone tests are more common for small animal studies (44) but are also reported for humans (45). At the apparent tissue level, cores of cancellous bone harvested from the vertebrae, proximal femur, and tibia are loaded to fracture and the resulting load and deformation values, representative of the core's microarchitectural and material characteristics, are converted to stress and strain and used to provide estimates of strength and toughness. At the material or matrix level, a variety of tests are available that utilize machined specimens with or without notches. The testing of specimens without a notch relies on the presence of a random flaw in bone that grows into a

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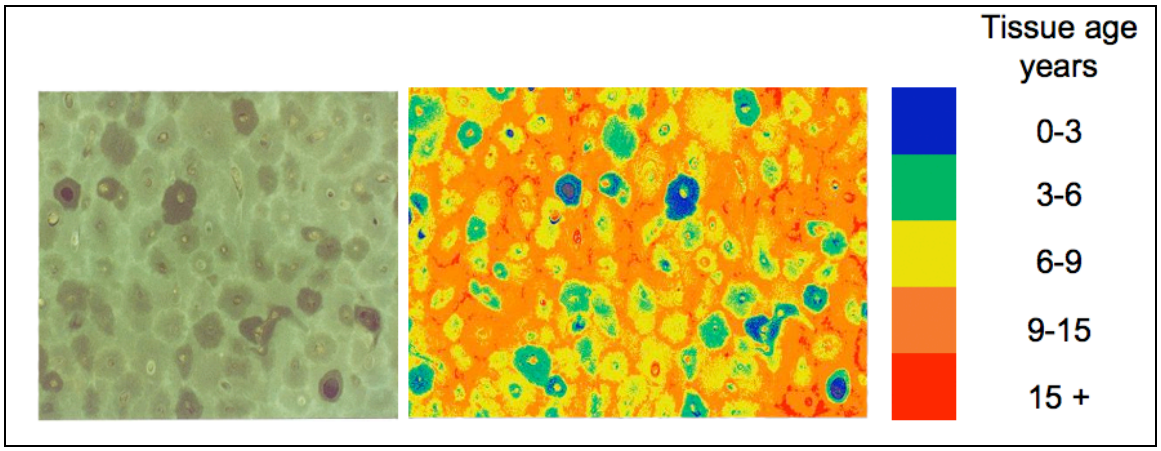

Fig. 4. Fluorescence map of a cortical bone cross-section indicating the distribution of AGEs in bone. The micrograph on the left shows an image collected using the excitation/emission settings corresponding to the fluorometric assay (excitation/emission: 370/440 nm). The micrograph on the right shows a false color image based on the fluorescence intensity of interstitial bone in bones of different ages. Similar to the fluorometric assay (34), this technique provides the measurement of AGEs within individual microstructural components of bone in terms of fluorescence normalized to a quinine sulphate standard (37). Images and analysis provided by Gary Gibson, PhD (Henry Ford Hospital) based on Gibson *et al*. (37).

fracture and the resulting load and deformation values are converted into stress and strain to provide measures of strength and toughness (46). The testing of the notched specimen relies on a machined flaw in bone, representing a microcrack or other weakness. Under loading the flaw grows into a fracture and the resulting load, crack length and displacement data are used to provide measures of bone's resistance to initiation and propagation of fracture (39). As whole bone strength is considered to be a measure of fracture risk in bone (43), the material level tests may seem irrelevant while assessing the effects of AGEs on bone fracture. However, AGEs are material level modifications and the experience from the aircraft industry and engineering materials shows that material level changes can cause a crack to grow into a full-scale failure of the entire structure including bridges and fuselages.

Contribution of AGEs to Bone Fracture

Accumulation of AGEs in bone with aging (15;17;40) and diabetes (8;44) and the concurrent increase in bone fracture risk (47-48) have led to a number of investigations of the contributions of AGEs to bone fracture. These investigations have either tested the correlation between AGE levels and bone fracture or have used *in vitro* models to evaluate the causality between accumulation of AGEs and bone fracture.

Correlation approaches relate AGEs to clinically assessed bone fracture or biomechanically determined bone fracture properties. For example, pentosidine, measured from urine or serum, predicts vertebral fractures in postmenopausal women and older adults with diabetes (33;48). Furthermore, after adjustment for traditional risk factors, urinary pentosidine predicts vertebral fracture in the general population (49). However the level of correlation and its significance varies among different cohorts due to differences in dietary habits, HPLC methods and the contribution of other tissues to secreted pentosidine (32).

In contrast to clinical data, cadaveric models are equivocal on the strong correlation between increased AGEs and bone fracture. Saito *et al*. (50;51) have shown that, compared to age-matched controls, pentosidine levels are elevated in cortical and cancellous bone tissue excised from hip fracture patients. Viguet-Carrin *et al*. (45) have shown that bone's pentosidine predicts the whole bone fracture properties of human vertebrae independently of BMD. At the

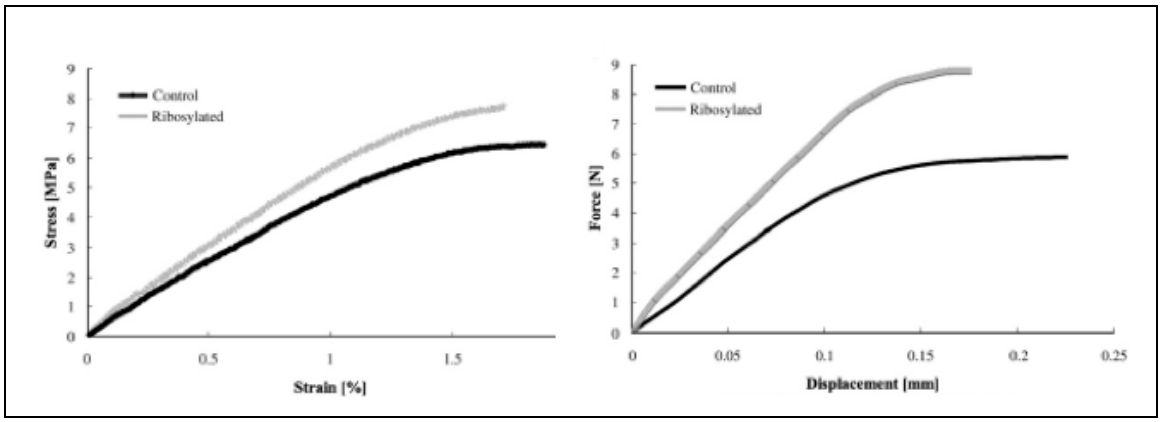

Fig. 5. Representative curves of mechanical tests conducted at apparent tissue (left) and matrix (trabecular) levels of human cancellous bone. Note that the average strength of *in vitro* ribosylated cancellous bone is 7% higher than its matched control but the average toughness is 48% lower (35). With permission from Elsevier (35).

apparent level, AGE content, measured by the fluorometric assay (35) and HPLC (52), predicts the post-yield fracture properties of cancellous bone obtained from the human femoral head and vertebral bodies (age range: 42 to 97 years). Similar reports are available at the matrix level where post-yield fracture properties measured from individual trabeculae (35;52) and cortical bone specimens (40;53) (age range 42 to 90 years) were predicted by AGEs.

In vitro NEG and animal models showing the accumulation of AGEs in bone have more directly implicated AGEs as one of the causes of increased bone fragility. The induction of NEG via *in vitro* ribosylation resulted in dose-dependent AGE accumulation in bone and caused a consequent reduction in bone's matrix- and apparent-level fracture properties (34;35). Furthermore, AGE-induced stiffening of the organic matrix was established as one of the mechanisms of AGE-induced bone fragility (34). Similar findings were reported from animal models where the accumulation of AGEs in type 2 (adult) diabetes led to a consequent increase in bone fragility (8;44).

More importantly, some of the changes in bone matrix due to antiresorptive postmenopausal osteoporosis therapy with bisphosphonates mirror the results presented above. In particular, bisphosphonate therapy results in the accumulation of AGEs in bone in dogs (54) and postmenopausal women (55). Furthermore, accumulated AGEs due to bisphosphonate therapy correlate with reduced bone turnover rates and increased bone fragility (36;55). Because a number of bone matrix changes accompany bisphosphonate therapy (56), further work is necessary to determine the mechanism of AGE accumulation with bisphosphonate therapy and the extent to which bisphosphonate-induced increases in AGEs contribute to bone fragility.

Conclusions

Similar to tissues with slow turnover, NEG occurs in bone leading to the accumulation of AGEs. Out of several potential intermolecular AGE crosslinks only one, pentosidine, has been identified in bone. Thus current methods of estimating AGEs in bone use either an HPLC technique to measure pentosidine or a fluorometric assay to measure the normalized bulk fluorescence of the organic matrix. Studies done at whole bone, apparent tissue and matrix levels have shown a negative relationship between AGE content and bone fragility. The use of *in vitro* glycation models and diabetic animals has helped to establish causality between the accumulation of AGEs and the increased propensity of bone to fracture. Information obtained from cadaveric and animal models on the mechanism of AGE accumulation and its effects on bone fragility is already proving to

be relevant and useful in a clinical setting. Several approaches are currently being tested to determine the efficacy of AGEs, measured from serum or urine, as valid markers of bone fracture risk. Accumulation of AGEs and their correlation with increased bone fragility has also been shown to occur during bisphosphonate therapy for postmenopausal osteoporosis.

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