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Research Paper

A direct role of collagen glycation in bone fracture

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ABSTRACT

Non-enzymatic glycation (NEG) is an age-related process accelerated by diseases like diabetes, and causes the accumulation of advanced glycation end-products (AGEs). NEG-mediated modification of bone's organic matrix, principally collagen type-I, has been implicated in impairing skeletal physiology and mechanics. Here, we present evidence, from in vitro and in vivo models, and establish a causal relationship between collagen glycation and alterations in bone fracture at multiple length scales. Through atomic force spectroscopy, we established that NEG impairs collagen's ability to dissipate energy. Mechanical testing of in vitro glycated human bone specimen revealed that AGE accumulation due to NEG dramatically reduces the capacity of organic and mineralized matrix to creep and caused bone to fracture under impact at low levels of strain (3000–5000 μstrain) typically associated with fall. Fracture mechanics tests of NEG modified human cortical bone of varying ages, and their age-matched controls revealed that NEG disrupted microcracking based toughening mechanisms and reduced bone propagation and initiation fracture toughness across all age groups. A comprehensive mechanistic model, based on experimental and modeling data, was developed to explain how NEG and AGEs are causal to, and predictive of bone fragility. Furthermore, fracture mechanics and indentation testing on diabetic mice bones revealed that diabetes mediated NEG severely disrupts bone matrix quality in vivo. Finally, we show that AGEs are predictive of bone quality in aging humans and have diagnostic applications in fracture risk.

1. Introduction

Bone matrix is a composite of mainly type-I collagen and mineral, and smaller quantities of non-collagenous proteins (Zylberberg, 2004). The ability of bone to resist fracture is determined not only by bone mineral density, as previously thought, but also by the quality of its organic extracellular matrix (Hernandez and Keaveny, 2006; Burr, 2002). Type-I

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collagen, which comprises over 90% of the organic matrix, imparts ductility and toughness to bone. Collagen is built of tropocollagen triple helical molecules that self assemble into larger fibrils, a few hundred nanometers in diameter, and exhibit the characteristic 67 nm D-periodicity (Wang et al., 2001). Self-assembly of collagen involves the formation of systematic enzymatic crosslinks such as pyrrole and pyridinoline, (Knott and Bailey, 1998; Viguet-Carrin et al., 2006). Enzymatic collagen cross-links mature up to 15 years of age (Eyre et al., 1988; Saito et al., 1997) and are instrumental in providing collagen the necessary stability and mechanical competence to resist deformation. Various studies (Shen et al., 2008; Gutsmann et al., 2004; Graham et al., 2004) have demonstrated that fibrils undergo periodic molecular deformation and stretching under force, which results in energy dissipation and retardation of crack growth within the bone matrix (Buehler, 2007; Gupta et al., 2006; Zimmermann et al., 2011).

Aging or diseases like diabetes cause collagen type-I to crosslink through non-enzymatic glycation (NEG), resulting in the formation of advanced glycation endproducts (AGEs) (Vashishth, 2007; Saito and Marumo, 2013). NEG-mediated crosslinking involves a reaction between an aldehyde of a reducing sugar (glucose or ribose) and amino groups of lysine or hydroxylysine present on collagen. The resultant aldimine complex rearranges to form a Schiff base or Amadori product, which subsequently undergoes reactions with other amino groups to form AGE crosslinks (Vashishth, 2009). AGEs can form within the fibril and between individual collagen fibrils, and their number can increase up to five times with age (Sell and Monnier, 1989; Odetti et al., 2005). They have been correlated to reduced bone toughness (Vashishth et al., 2001; Wang et al., 2002; Garnero et al., 2006). Non-enzymatic glycation and AGE accumulation, due to aging, not only deteriorate bone quality and material properties (Vashishth et al., 2000; Karim and Vashishth, 2012), but also increase stiffness and brittleness in other musculoskeletal tissues like cartilage (Chen et al., 2002) and tendon (Reddy, 2004). In spite of our growing understanding of glycation in bone tissue, there is no evidence to establish the mechanism by which molecular level modifications of bone collagen impair energy dissipation of bone and cause fracture. Furthermore, from a clinical perspective, it is unknown if NEG alters bone's response to suddenly applied impact loading, typical of falls, and cause it to fracture. Cortical bone bears impact of loading during fracture but it is not established if effect of NEG is ubiquitous across age and in diseases such as diabetes that, despite higher bone mineral density, show increased incidence of bone fracture (Vestergaard, 2007).

In this study, we hypothesized that glycation results in matrix level modifications across the scales of hierarchy in bone matrix, and these modifications cause, and are not simply correlative to, the reduction in bone mechanical properties observed in previous studies. Our objective was to use both, in vitro, and in vivo tests, to evaluate the hypothesis. Specifically, using in vitro mechanical studies on glycated collagen, glycated human bone specimens and their age matched controls, and in vivo diabetic animal models, we show how AGE accumulation in bone collagen causes an impairment in biomechanical properties. The use of age matched controls allows us to attribute NEG (glycation doubles AGE after 7 days of treatment, equivalent to 30 yr of aging) as a cause of decreased bone fragility.

2. Materials and methods

2.1. AFM studies on collagen type-I

Collagen fibrils were produced in-vitro using dissolved rat tail collagen (BD Biomedicals) and 1X phosphate buffered saline (PBS). The collagen solution was warmed up to room temperature and titrated with 1X PBS to achieve a physiological pH of 7.4. This procedure was followed by incubation of the resulting mixture for 18 h at 37 °C to allow formation of collagen fibrils. After their formation, the fibrils were centrifuged and removed as control specimen, or glycated using 1.5 M ribose solution. Samples were dried on a glass cover slip at 37 °C and imaged using MFP 3D atomic force microscope (Asylum Research, Santa Barbara, CA) and AC 160 cantilevers (Asylum Research, $k=40$ N/m, $f_o=300$ kHz). Fibril diameter (D) was measured using a section analysis tool ($N=8$) (Igor). Force spectroscopy using cantilever approach and retraction rates of 5 μm/s were carried out in PBS under ambient conditions using Olympus Biolever cantilevers ($k=0.025$ N/m). An intermittent dwell time of 4 s was incorporated to facilitate binding between the AFM tip and fibrils on sample surface. Area under the force-distance curves, a combined measure of interaction between collagen fibrils and tip-fibril interaction, was quantified ($N=5$). Our peak forces and extensions were similar to those reported in other studies on collagen type-I (Bozec and Horton, 2005; Gutsmann et al., 2004).

2.2. In vitro non-enzymatic glycation

Aliquots (10 ml) of the precipitated collagen were sealed in dialysis tubing with a pore size of 300 nm. These were placed in ribosylation and control solutions for a period of 7 days at 37 °C and pH was maintained between 7.2 and 7.4. The ribosylation solutions contain 1.5 M of ribose, protease inhibitors to prevent enzymatic reactions (25 mM ε-amino-*n*-caproic acid, 5 mM benzamidine, 10 mM *N*-ethylmaleimide) and 30 mM HEPES in Hanks buffer (Vashishth, 2007). The control solution had the same composition as the ribosylation solution but contains no ribose.

2.3. AGE assay

For all samples, total fluorescent AGEs were measured by a fluorometric assay (Vashishth et al., 2001). Cortical bone samples were freeze-dried and hydrolyzed in 6 N HCl for 20 h at 110 °C to obtain sample hydrosylates. Hydrosylates were centrifuged at 12,000 rpm for 30 min and the supernatant was obtained. Using an Infinite 200 microplate reader (Tecan; 2450 Zanker Road, San Jose, CA 95131, USA) fluorescence was measured for the hydrosylate supernatants and normalized against serially diluted quinine standards (stock: 10 μg/mL quinine per 0.1 N sulfuric acid) at 360/460 nm (excitation/emission). For the hydroxyproline measurements, chloramine-T, perchloric acid (3.15 M) and *p*-dimethylaminobenzaldehyde (PDB) solutions were made immediately before