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COMMENTARY

Effects of estrogen depletion and drug treatment on collagen microstructure: implications

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Although much attention is paid to the relationship of bone's mineral to fragility and fracture, collagen's role is often overlooked. Although water and non-collagenous proteins have important roles in the bone,[1] the bulk of the tissue is made up of a flexible organic matrix (~90% Type I collagen) that is reinforced by an apatitic mineral phase in a manner similar to reinforced concrete. The mechanical integrity of bone is derived from the complex interplay between these nanoscale constituents, their disparate mechanical properties and their spatial organization.[2–4] Collagen is responsible for bone's ductility and toughness, properties essential for fracture resistance.[5,6] Collagen is also the primary driver of tensile strength and, as long bones are loaded in bending and fail under tension, collagen quality is critical to reducing fractures. Despite these important characteristics, we have a limited understanding of how alterations to collagen translate to bone fragility.

The nanostructural and microstructural organization of collagen are critically important. In species that undergo osteonal remodeling, the most common microstructural elements are circumferential and osteonal lamellar bone. As early as 1905, the idea of a twisted plywood-like structure of mineralized collagen in bone was postulated.[7] This model was extended in the 1980s with the idea that fibril arrays change their orientation from layer to layer in osteonal lamellae.[8] Several models have tried to explain the organization of lamellar bone; however, conflicting observations remain. Recent work by Reznikov *et al.*[9] postulated the presence of two distinct materials within the human lamellar bone, one ordered with two major preferred directions, and one disordered that envelops the ordered components and the space in between. Considering the role it may have in crack propagation and fracture toughness, a complete understanding of collagen's normal microstructure and changes that occur with disease or therapeutic treatment is long overdue.

Cauble *et al.* report the use of atomic force microscopy to probe the nanoscale structure of collagen and how drug treatments may influence collagen organization. The authors demonstrate that, in processed samples of rabbit cortical bone, estrogen depletion is associated with a disorganized collagen microstructure, a change that is largely prevented by a cathepsin-K inhibitor. Partial prevention of these organizational changes was attained with alendronate and estradiol treatment. It was recently shown that CatK degrades the proteoglycan (PG) network surrounding collagen fibers before the disassembly of those fibers into fibril bundles and, ultimately, their final degradation.[10] The authors hypothesize that CatK-induced degradation of PGs during resorption perturbs the interactions between adjacent fibrils, resulting in the microstructural disorganization noted in the OVX samples. This observation is interesting but should be interpreted with caution. It is possible that the observed changes are more fully explained by an interaction between the collagen structure and sample processing. Part of the process to expose collagen involves demineralization with EDTA followed by sonication. EDTA alone is unlikely to directly affect collagen but, as mineral is removed from the fibrils, changes do occur including a shift in D-spacing.[11] If there are chemical differences in mineral between the different treatment groups, the amount of mineral removed may differ even if the specimens are demineralized in the same way. This could lead to an interactive effect with sonication, producing disordered collagen that varies according to the amount of stabilizing mineral that has been eliminated. Alternatively, if PG degradation weakens the bond between collagen and mineral so that OVX has more mineral removed, sonication could indirectly lead to increased disorder. If, as the authors hypothesize, cleavage of PGs occurs because of CatK without much cleavage of the collagen protein structure itself, fibrils could be more sensitive to sonication. The observed disorder would still be interesting as a marker of disease and treatment but may not directly bear on the impact of estrogen depletion or antiresorptive drug treatment on the quality of intact bone.

The functional relevance of these observations remains to be addressed. Although 15 regions were interrogated, these were limited to 1 cm of the bone's length in a predominantly cortical portion of the mid-diaphysis in a single anatomical quadrant.

A recent study used second harmonic generation to map collagen organization over much larger areas and demonstrated regional differences,[12] and such a technique may be more informative. It would be particularly interesting to examine trabecular regions. Ideally, studies of collagen structure would be related to regions of bone degradation, formation or quiescence. The authors have made an initial step in relating the collagen structure to factors that modulate remodeling. However, more investigation is needed to understand the mechanism by which these changes in organization could come about and what they mean both biologically and in terms of mechanical function.

In the model employed by the authors, estrogen withdrawal accelerates bone remodeling with a bias toward resorption, mediated by increased RANK-ligand activity. It is not entirely clear whether the altered collagen described by the authors is found at remodeling sites that were active at the time of euthanasia, or whether it was from areas that were remodeled during the treatment period. In the former case, the collagen fibrils of interest could be in the process of degradation, as proposed by the authors, or in the process of formation. This distinction is of considerable importance with regard to the implications of the reported findings.

The proposed mechanism whereby 'oblique' collagen fibrils are produced is the degradation of PGs surrounding the fibrils, without apparent damage to the collagen itself. Presumably, the absence of the PG network around the collagen fibrils would make them more susceptible to the effects of sonication during the sample preparation process. Panwar *et al.*[10] have demonstrated that the action of CatK is initially on PG; however, the progressive degradation process continues and completely breaks down the collagen as well. Thus, the proposed mechanism may not be fully explanatory. If the initial degradation of PGs did leave the observed fibrils intact, they would be expected to be found almost entirely at sites where resorption was active at the time of euthanasia, not in the bone formed earlier in the course of the estrogen-deprived or drug-treated phase of the experiment, nor in the bone that was mainly under active formation during euthanasia. The samples are from largely cortical areas, in which the proportion of bone under active resorption at the time of euthanasia would ordinarily be relatively small. The authors' proposed mechanism leads to the inference that the less orderly bone is a marker for the rate of degradation by CatK. This would be consistent with the intermediate effects of estradiol and alendronate, which decrease bone remodeling but do not specifically interfere with the action of CatK. It would be useful to know whether the areas of active resorption could fully account for the proportion of 'oblique' fibrils described. The functional significance *in vivo* is unclear, especially if the disorder only becomes apparent after sonication.

Alternatively, the less orderly collagen might have been formed during the experimental exposure period. This hypothesis implies that acceleration of the remodeling process could result in the formation of subtly altered collagen in an osteoporosis model. Gross alterations of collagen structure have been demonstrated in Paget's disease, in which rapid, disorderly bone formation is driven by markedly accelerated resorption in localized areas. Effective treatment of Paget's disease with bisphosphonates results in a return to normal-appearing lamellar bone formation, even in affected areas.[13] Could reduced turnover in the author's model result in minimization of disorder at the nanoscale? Could there be a specific effect of CatK that might account for an even greater effect versus other antiresorptives? Specific products of CatK degradation of collagen could have a role in the regulation of bone formation.[14] CatK may be expressed in cells of the osteoblast/osteocyte lineage, as well as in osteoclasts, adding another dimension to its potential regulatory role.[15]

The key point is whether this difference in uniformity of collagen exists in living unprocessed bone. If so, then what are the functional consequences? A high degree of uniformity might be detrimental from the perspective of toughness; however, other aspects of bone strength may dominate in intact animals. Is it plausible that, in the drug-treated case, there is sufficient restoration of mass to reduce overall fragility, but this is partly offset in cortical bone by 'enhanced' uniformity? This might result in a net reduction in total fracture risk but with a shift in the anatomic pattern of fracture susceptibility toward cortical regions.

If the authors' interpretation of their results is correct, that the effect described is on the degradation of collagen, it is of interest but the functional implications are uncertain. If there is an effect on the formation of collagen and thus on the structural collagen in living bone, the effects of pharmacological intervention on collagen order may be of considerable importance. This critical question requires correlation with histology and perhaps other methods to identify the stage at which the variability in collagen order occurs or is prevented.

Conflict of Interest

HGB has acted as a consultant to Merck and Amgen and has received research funding from Merck and Amgen. The remaining author declare no conflict of interest

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