Differential Effects Between the Loss of MMP-2 and MMP-9 on Structural and Tissue-Level Properties of Bone

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ABSTRACT

Matrix metalloproteinases (MMPs) are capable of processing certain components of bone tissue, including type 1 collagen, a determinant of the biomechanical properties of bone tissue, and they are expressed by osteoclasts and osteoblasts. Therefore, we posit that MMP activity can affect the ability of bone to resist fracture. To explore this possibility, we determined the architectural, compositional, and biomechanical properties of bones from wild-type (WT), *Mmp2*^{-/-}, and *Mmp9*^{-/-} female mice at 16 weeks of age. MMP-2 and MMP-9 have similar substrates but are expressed primarily by osteoblasts and osteoclasts, respectively. Analysis of the trabecular compartment of the tibia metaphysis by micro-computed tomography (μ CT) revealed that these MMPs influence trabecular architecture, not volume. Interestingly, the loss of MMP-9 improved the connectivity density of the trabeculae, whereas the loss of MMP-2 reduced this parameter. Similar differential effects in architecture were observed in the L₅ vertebra, but bone volume fraction was lower for both *Mmp2*^{-/-} and *Mmp9*^{-/-} mice than for WT mice. The mineralization density and mineral-to-collagen ratio, as determined by μ CT and Raman microspectroscopy, were lower in the *Mmp2*^{-/-} bones than in WT control bones. Whole-bone strength, as determined by three-point bending or compression testing, and tissue-level modulus and hardness, as determined by nanoindentation, were less for *Mmp2*^{-/-} than for WT bones. In contrast, the *Mmp9*^{-/-} femurs were less tough with lower postyield deflection (more brittle) than the WT femurs. Taken together, this information reveals that MMPs play a complex role in maintaining bone integrity, with the cell type that expresses the MMP likely being a contributing factor to how the enzyme affects bone quality. © 2011 American Society for Bone and Mineral Research.

KEY WORDS: NANOINDENTATION; RAMAN; μ CT; MATRIX METALLOPROTEINASE; COLLAGEN; MINERAL

Introduction

Even though type 1 collagen is a critical determinant of fracture resistance,⁽¹⁾ there is an incomplete understanding of how genes that are important to organization of the organic matrix regulate material properties of bone tissue (ie, bone quality). Matrix metalloproteinases (MMPs) are a family of proteolytic enzymes that collectively degrade all components of the organic matrix of bone, and several MMP family members have the ability to process both helical and denatured type 1 collagen, including the closely related gelatinases MMP-2 and

MMP-9.⁽²⁾ To the best of our knowledge, comparisons between these two gelatinases have not been reported for their individual effects on the architectural, compositional, and biomechanical properties of bone.

Since MMPs are expressed by bone cells, there are a number of possibilities for how they could influence the strength and toughness of bone. For example, osteoclasts express several MMPs, including MMP-9.^(3–5) The generation of *Mmp9* null mice provided insight into how this osteoclast-derived MMP is involved in osteoclast recruitment to ossification centers during development. *Mmp9*^{-/-} long bones were 10% shorter than

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bones from wild-type (WT) mice,⁽⁶⁾ suggesting MMP-9 has a structural influence on the biomechanical properties of whole bones. Other studies indicate important roles for MMPs in osteoblast retraction and remodeling of nonmineralized osteoid,^(7,8) and certain osteoblast-derived MMPs such as MMP-13 are involved in the remodeling process and the mediation of osteoclastic bone resorption under the control of parathyroid hormone (PTH).^(9,10) Osteoblasts also express other MMPs, including MMP-2. The generation of *Mmp2* null mice provided insight into how this osteoblast-derived MMP affects the development of canaliculi.⁽¹¹⁾ *Mmp2*^{-/-} bones were reported to have lower bone mineral density (BMD) than WT bones with no apparent difference in the length, suggesting MMP-2 has a compositional influence on the biomechanical properties of bone.⁽¹¹⁾ Indeed, we recently observed that the degree of mineralization of the midshaft strongly correlated with estimated bending strength of the femur across WT and *Mmp2*^{-/-} mice (male and female).⁽¹²⁾ Thus MMP-2 and MMP-9 appear to play important yet poorly understood roles in bone formation and resorption that may influence whole-bone and material-level biomechanical properties.

Independent of their matrix processing functions, MMPs also may influence bone turnover and tissue remodeling via the processing of noncollagenous proteins such as growth factors and cytokines. For example, MMP-2 and MMP-9 have been shown to regulate the bioavailability and bioactivity of transforming growth factor β (TGF- β) by processing the proteins that sequester TGF- β in a latent state from its latent binding proteins.^(13,14) TGF- β is one of the few factors known to regulate tissue-level properties.^(15–17) When TGF- β signaling was decreased or increased in transgenic mouse models, the modulus and hardness of the tissue increased or decreased, respectively, as determined by nanoindentation.⁽¹⁵⁾ Therefore, modulation of MMP activity also may influence mechanical properties via indirect effects on TGF- β activity in addition to direct effects on matrix processing.

Given their role in bone matrix turnover and activation of growth factors, we posit that MMP-2 and MMP-9 also may influence the biomechanical properties of bone. This supposition is based on (1) the roles for MMP-2 and MMP-9 in processing type 1 collagen and type 1 collagen derivatives that are critical for the ability of bone tissue to dissipate energy as it fails (ie, bone toughness) and (2) their roles in governing the bioavailability and/or activity of factors affecting the recruitment of osteoclasts and the differentiation of osteoblasts. To test whether MMP-2 and MMP-9 potentially could affect bone quality, we characterized the structural, architectural, compositional, and biomechanical properties of bones from WT mice and genetic knockout mice lacking either MMP-2 or MMP-9.

Material and Methods

Tissue harvesting and processing

Female WT MMP-2-deficient mice (*Mmp2*^{-/-}) and MMP-9-deficient mice (*Mmp9*^{-/-}) on an FVB background ($n = 8$ to 10/genotype) were euthanized at 16 weeks of age following an approved Institutional Animal Care and Use Committee protocol.

Cleaned of soft tissue, the left femurs and lumbar vertebrae were stored in PBS at -20°C until imaging by micro-computed tomography (μCT), measuring of the axial length with digital calipers, and biomechanical testing. The left tibias were stored in 70% ethanol (EtOH) at 4°C . After μCT imaging, the bones were dehydrated through increasing concentrations of EtOH and embedded in methyl methacrylate (Fisher Scientific, Pittsburgh, PA, USA), *N*-butyl methacrylate (Sigma, St. Louis, MO, USA), methyl benzoate (Fluka, Analytical, Sigma, St. Louis, MO, USA), and polyethylene glycol 400 (Fluka).⁽¹⁸⁾

μCT analysis

To assess phenotypic differences in the architecture and structure of bone, each femur or L₅ vertebra was imaged using a $\mu\text{CT}40$ scanner (Scanco Medical, Brüttisellen, Switzerland) calibrated against a hydroxyapatite (HA) phantom. The long axis of each femur and tibia or the craniocaudal axis of the L₅ vertebra was aligned with the scanning axis. Cross-sectional images of the regions of interest were acquired with an isotropic voxel size of $12\text{ }\mu\text{m}$ at 55 kVp and 145 mA, 500 projections per 180-degree rotation, and an integration time of 300 ms. The regions of interest were (1) cortex of the femur at the midpoint (2 mm in length), (2) trabeculae within the proximal metaphysis of the tibia (0.24 to 1.20 mm below the growth plate), and (3) the trabeculae of the L₅ vertebral body (1.2 mm between end plates). After reconstruction, bone tissue was segmented from air or soft tissue such that each region of interest had unique thresholding parameters that were held constant across the genotypes.

Scanco evaluation software was used to quantify mean cortical thickness (Ct.Th), medullary volume (Ma.V), cortical porosity (Ct.Po), average cross-sectional area (Ct.Ar), and average moment of inertia with respect to the orientation of three-point bending test (I_{min}). Standard architectural characteristics of trabecular bone within the metaphysis and vertebral body were measured, including bone volume fraction (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), and connectivity density (Conn.D). The average volumetric mineral density of the mineralized tissue (TMD in mgHA/cm^3) also was measured for both the trabecular and cortical compartments.

Flexural testing

To determine the differences in biomechanical properties, each hydrated femur was placed on the lower support points of a three-point bending fixture with the anterior side down (ie, bending about the mediolateral plane). The span between the lower supports was 7 mm, and the load rate was 3.0 mm/min. Forces from a 100-N load cell (Honeywell, Morristown, NJ, USA) and displacements from the linear variable differential transducer (Dynamight 8841; Instron, Canton, OH, USA) were recorded at 50 Hz during the monotonic load-to-failure test (Fig. 1). Biomechanical properties that were assessed included the stiffness δ , which is the slope of the linear portion of the force-displacement curve; the peak load P_{max} , which is the maximum force endured by the mid-diaphysis; the yield load P_y , which is the force at the proportional limit; the postyield deflection (PYD), which is the displacement from yield to fracture; and the toughness, which is the area under the force-displacement curve.

Fig. 1. Typical force-deflection curves are from the three-point bending tests of WT, *Mmp2*^{-/-}, and *Mmp9*^{-/-} femurs.

generated by the testing divided by $2 \times Ct.Ar$. Yielding was deemed to occur when the secant stiffness was 15% less than the initial stiffness.⁽¹⁹⁾ Material properties of modulus and strength were estimated for the mid-diaphysis of the femur using standard beam theory that accounts for the effect of structural geometry on force and displacement values using I_{min} and the distance between the neutral axis of bending and the outermost point in the anteroposterior direction (C_{min}).⁽²⁰⁾ The previously described μ CT scans provided these structural characteristics.

Confocal Raman spectroscopy

Raman spectroscopy (RS) is an optical technique sensitive to specific Raman active vibrational modes of molecules within a sample and was used here to determine whether differences existed in physicochemical properties (ie, tissue composition) at the micron length scale between the genotypes. A cross section of the polymethylmethacrylate-embedded tibia was cut at the midshaft using a diamond-embedded circular saw (South Bay Technology, San Clemente, CA, USA) to expose the bone tissue at the midshaft. A lapping wheel (Exakt Technologies, Oklahoma City, OK, USA) then was used to grind the exposed surface on successive grits of silicon carbide paper (800, 1200, and 4000), followed by polishing with 0.1- and 0.05- μ m alumina suspensions on a synthetic cloth.

After securing the bone specimen to the stage of a confocal Raman microscope (Ramanscope Mark III; Renishaw, Hoffman Estates, IL, USA), a $\times 50$ objective focused the light from a 785-nm laser diode source (Innovative Photonic Solutions, Monmouth Junction, NJ, USA) to an approximate 4- μ m spot just below the surface of the bone tissue. Each Raman spectra was acquired through a 35- μ m slit by the Renishaw spectrograph and had a spectral resolution of 1 cm^{-1} . There were three accumulations of the Raman scatter with an integration time of 10 seconds and a binning of 3 across the wave numbers. The wave-number axis of the spectrograph was calibrated regularly with a silicon reference (peak at 520 cm^{-1}) such that the strongest intensities were collected by the charge-coupled device (CCD) of the spectrograph. Nine spectra were collected from the triangular cortex of the diaphysis per tibial cross section (three per side).

After subtracting the background fluorescence using a polynomial fitting algorithm,⁽²¹⁾ each Raman spectrum (see Supplemental Fig. 15 for an example) provided quantitative assessments of specific peaks or peak ratios to provide average compositional properties per tibia. Specifically, we determined the compositional properties as follows: (1) ν_1 phosphate (ν_1PO_4) peak intensity (at 960 cm^{-1}) per amide III peak intensity (at 1248 cm^{-1}) and ν_1PO_4 per CH_2 -wag peak intensity (at 1452 cm^{-1}) gave two measures of the mineral-to-collagen ratio,⁽²²⁾ carbonate peak intensity (at 1072 cm^{-1}) per ν_1PO_4 peak intensity gave carbonate substitution (loss of ideal crystal structure),⁽²³⁾ and the width at half the maximum of ν_1PO_4 peak intensity gave crystallinity (mineral maturity).⁽²⁴⁾

Nanoindentation

Whole-bone mechanical tests do not necessarily provide a measurement of material properties that are entirely independent of bone structure and microscopic porosity.⁽²⁵⁾ Therefore, to determine the phenotypic differences in the tissue-level properties (independent of size), the same specimens used in the RS assessment were analyzed by nanoindentation, which measures modulus and hardness from the tissue at the micron length scale. Once the specimen of dry bone was mounted within the nanoindentation system (MTS XP, Eden Prairie, MN, USA), a Berkovich diamond tip (a pyramidal-shaped indenter with a center line-to-face angle of 65.3 degrees) was driven into the surface of the bone section using the following scheme: (1) load at a constant loading rate chosen to reach a maximum depth of 1 μ m in 30 seconds, (2) hold at P_{max} for 10 seconds (to minimize the effects of viscoelastic deformation and creep of the specimen), (3) unload at the same rate used in the first loading step to 10% of P_{max} , (4) hold the indenter on the surface for 60 seconds to establish thermal drift, and (5) completely remove the indenter from the bone surface. From the resulting force-displacement curve (Fig. 2), the elastic modulus E and hardness H of the tissue at the point of indentation (0.25- μ m resolution) were calculated following the method of Oliver and Pharr.⁽²⁶⁾ This requires an initial calibration procedure using fused silica to establish the relationship between depth of indent and contact area A_c of the tip.

Fig. 2. Examples of force-displacement curves are from nanoindentation tests on the cross sections of WT, *Mmp2*^{-/-}, and *Mmp9*^{-/-} tibias.

Statistical analysis

Differences among the genotypes in each property were determined using one-way ANOVA at $p < .05$, and statistically significant differences between WT and *Mmp2*^{-/-} bones or between WT and *Mmp9*^{-/-} bones were determined using the post-hoc Holm-Sidak method of multiple comparisons versus a control (overall significance level = 0.05). In the event that the normality test failed, the nonparametric Kruskal-Wallis ANOVA on Ranks and Dunn's post-hoc method of multiple comparisons versus a control were used ($p < .05$). All statistical analyses were performed with SigmaStat (Version 3.5; SYSTAT Software, Inc., Chicago, IL, USA).

Results

MMP-2 and MMP-9 differentially contribute to trabecular architecture and density

Trabecular architecture is thought to contribute to the risk of osteoporotic fracture,⁽²⁷⁻³⁰⁾ and the loss of both MMP-2 and MMP-9 was found to affect several characteristics of architecture. Interestingly, the loss of neither MMP-2 nor MMP-9 had any appreciable effect on trabecular bone volume fraction (BV/TV) within the metaphysis of the tibia (Table 1). However, while *Mmp9*^{-/-} bones had more trabeculae, they typically were thinner than WT trabeculae in the tibial metaphysis. This difference in trabecular architecture also was reflected in trabecular connectivity density (Conn.D), with trabeculae in *Mmp9*^{-/-} mice having a higher Conn.D than WT controls. In contrast, *Mmp2*^{-/-} mice had fewer trabeculae and lower Conn.D than WT mice (Table 1). The mineral density of the trabeculae (Tb.TMD) was less in *Mmp9*^{-/-} mice than in WT mice, but no difference was observed in Tb.TBMD between *Mmp2*^{-/-} and WT mice (Table 1).

As for the L₅ vertebral body (VB), both *Mmp2*^{-/-} and *Mmp9*^{-/-} mice had lower BV/TV than WT mice (Table 1). Again, there were contrasting effects on trabecular architecture in that the loss of MMP-9 caused thinner trabeculae but increased Conn.D and the loss of MMP-2 caused fewer trabeculae and modest, non-significant reduction in Conn.D. There was a decrease in TMD of

VB trabeculae with the loss of both MMP-2 and MMP-9 (Table 1). Thus, while the loss of either MMP-2 or MMP-9 altered trabecular architecture, each MMP had differential effects on specific architectural parameters.

MMP-2 and MMP-9 differentially affect structural and biomechanical properties of the femur

Fracture resistance depends on the structure or distribution of tissue about the bending axis as well as the inherent quality of the tissue itself. Therefore, we determined whether structural and biomechanical differences existed between the WT and *Mmp* null groups. The *Mmp9*^{-/-} femurs were significantly shorter by 8% in length than the WT femurs, whereas the *Mmp2*^{-/-} femurs were significantly shorter by 5% than the WT femurs (Table 2). The diaphyseal cortex was nonsignificantly thicker by 9% for *Mmp9*^{-/-} mice, whereas it was significantly thinner by 23% for *Mmp2*^{-/-} mice, when compared with WT mice. There also were contrasting effects between the loss of the MMP-2 and the loss of MMP-9 in the cross-sectional moment of inertia I_{min} . Despite the thinner cortex, the I_{min} of *Mmp2*^{-/-} femurs was not significantly different from that of WT femurs (Table 2). The *Mmp9*^{-/-} femurs, however, had a significantly lower I_{min} than those of WT mice. Interestingly, *Mmp2*^{-/-} cortices had a substantially higher porosity than WT cortices (Table 2), and this Ct.Po tended to occur near the endosteal surface (Fig. 3).

Despite the lack of difference in I_{min} between *Mmp2*^{-/-} and WT femurs, the bending stiffness, yield force, and peak force all were less in the *Mmp2*^{-/-} mice than in the WT mice. In contrast, the *Mmp9*^{-/-} diaphysis, which had a reduced I_{min} , had nearly the same yield force and peak force as the WT diaphysis. The stiffness of the *Mmp9*^{-/-} diaphysis, however, was 10.5% less than that of WT diaphysis (Table 2). Of note, the body mass of *Mmp2*^{-/-} mice (21.4 ± 1.0 g) and the *Mmp9*^{-/-} mice (22.5 ± 1.2 g) was significantly less than that of WT mice (24.3 ± 1.3 g; $p < .001$ versus either genotype) at death.

When accounting for structure in the biomechanical properties using I_{min} or Ct.Ar, we found additional differential effects of the two MMPs on the estimated material properties. The *Mmp2*^{-/-} femoral diaphysis had a lower modulus and yield strength than the WT diaphysis, whereas the *Mmp9*^{-/-} diaphysis

Table 1. μ CT-Derived Measurements (Mean ± SD) of Trabecular Bone for Each Genotype

Property	Unit	Wild-type	<i>Mmp2</i> ^{-/-}	<i>Mmp9</i> ^{-/-}
Tibia metaphysis		<i>n</i> = 8	<i>n</i> = 9	<i>n</i> = 11
BV/TV	%	11.7 ± 2.3	10.0 ± 1.4	11.7 ± 1.5
Tb.Th	mm	0.0511 ± 0.0020	0.0528 ± 0.0032	0.0460 ± 0.0032*
Tb.N	1/mm	4.21 ± 0.34	3.27 ± 0.25*	4.69 ± 0.40*
Conn.D	1/mm ³	56.2 ± 19.5	36.0 ± 11.6*	81.2 ± 20.5*
Tb.TMD	mg HA/cm ³	1098 ± 27	1088 ± 16	1031 ± 15*
L5 vertebral body		<i>n</i> = 8	<i>n</i> = 9	<i>n</i> = 11
BV/TV	%	27.1 ± 2.2	23.8 ± 1.3*	23.9 ± 1.9*
Tb.Th	mm	0.055 ± 0.002	0.053 ± 0.001	0.051 ± 0.003*
Tb.N	1/mm	4.43 ± 0.25	3.83 ± 0.28*	4.38 ± 0.24
Conn.D	1/mm ³	199.1 ± 10.0	186.3 ± 16.7	284.4 ± 24.4*
Tb.TMD	mg HA/cm ³	1047 ± 9	1017 ± 11*	1022 ± 11*

*WT versus *Mmp2*^{-/-} or WT versus *Mmp9*^{-/-} was statistically significant at $p < .05$.

Table 2. Structural and Biomechanical Properties (Mean $\pm$ SD) of the Femur Diaphysis for Each Genotype

Property	Unit	WT	<i>Mmp2</i> ^{-/-}	<i>Mmp9</i> ^{-/-}
Femur diaphysis		<i>n</i> = 8	<i>n</i> = 9	<i>n</i> = 11
Length	mm	13.8 $\pm$ 0.2	13.1 $\pm$ 0.1*	12.7 $\pm$ 0.3*
Ct.Th	mm	0.214 $\pm$ 0.004	0.165 $\pm$ 0.005*	0.234 $\pm$ 0.019
Ct.Po	%	2.58 $\pm$ 0.12	4.56 $\pm$ 0.32*	2.52 $\pm$ 1.88
I _{min}	mm ⁴	0.107 $\pm$ 0.010	0.110 $\pm$ 0.006	0.086 $\pm$ 0.006*
Ct.Ar	mm ²	0.813 $\pm$ 0.042	0.700 $\pm$ 0.021*	0.840 $\pm$ 0.065
Ct.TMD	mg HA/cm ³	1481 $\pm$ 12	1367 $\pm$ 8*	1481 $\pm$ 15
Stiffness	N/mm	166 $\pm$ 7	122 $\pm$ 13*	149 $\pm$ 22*
Yield force	N	19 $\pm$ 2	12 $\pm$ 1*	19 $\pm$ 2
Peak force	N	22 $\pm$ 2	17 $\pm$ 2*	20 $\pm$ 2
PYD	mm	0.25 $\pm$ 0.08	0.32 $\pm$ 0.14	0.13 $\pm$ 0.05*
Modulus	GPa	11.3 $\pm$ 1.3	8.0 $\pm$ 0.7*	12.3 $\pm$ 1.7
Yield strength	MPa	199 $\pm$ 14	129 $\pm$ 17*	220 $\pm$ 16*
Toughness	N $\cdot$ mm/mm ²	4.0 $\pm$ 1.1	3.9 $\pm$ 1.0	2.4 $\pm$ 0.7*
L ₅ vertebral body		<i>n</i> = 6	<i>n</i> = 8	<i>n</i> = 11
Stiffness	N/mm	196 $\pm$ 59	153 $\pm$ 80	195 $\pm$ 73
Yield force	N	36.2 $\pm$ 9.5	22.9 $\pm$ 4.3*	32.1 $\pm$ 6.7
Peak force	N	40.1 $\pm$ 5.6	28.6 $\pm$ 6.4*	36.0 $\pm$ 4.3

*WT versus *Mmp2*^{-/-} or WT versus *Mmp9*^{-/-} difference was statistically significant at *p* < .05.

had a higher modulus and yield strength than the WT diaphysis, albeit the difference in the estimated moduli was marginal (Table 2). Interestingly, the stronger *Mmp9*^{-/-} cortical bone had less toughness and postyield deflection (ie, was more brittle) than the WT cortical bone. There was no statistically significant difference in these properties between *Mmp2*^{-/-} and WT femurs. Thus MMP-2 appears important to bone strength, whereas MMP-9 appears important to bone toughness.

MMP-2 and MMP-9 differentially influence the biomechanical properties of the vertebral body

The VB from the *Mmp2*^{-/-} mice had a lower stiffness, yield force, and peak force than the VB from WT mice when tested in compression (Table 2). There were moderate decreases in these

biomechanical properties with loss of MMP-9, but they were not statistically different.

MMP-2 and MMP-9 differentially affect tibial tissue-level properties

Since biomechanical tests of whole mouse bones can only estimate material properties of the tissue, we applied Raman spectroscopy and nanoindentation to transverse sections of the tibia. In the tibial diaphysis, there was a decrease in the mineral-to-collagen ratio in *Mmp2*^{-/-} mice but not in *Mmp9*^{-/-} mice compared with WT controls (Table 3). This was evident when the mineral peak was normalized by the amide III or CH₂-wag (ie, collagen peaks). There were trends but no statistically significant differences in the type B carbonate substitution (CO₃/PO₄)

Fig. 3. *Mmp2*^{-/-} diaphysis had a cortex with large pores near the endosteal surface, whereas *Mmp9*^{-/-} diaphysis had a solid cortex with a small periosteal circumference.

Table 3. Raman-Derived Measurements (Mean $\pm$ SD) of Cortical Bone Tissue Within the Tibia for Each Genotype

Property ^a	Unit	WT	<i>Mmp2</i> ^{-/-}	<i>Mmp9</i> ^{-/-}
Tibia diaphysis		<i>n</i> = 8	<i>n</i> = 9	<i>n</i> = 11
$\nu_1\text{PO}_4/\text{amide III}$	—	13.5 $\pm$ 0.8	12.0 $\pm$ 0.8*	13.4 $\pm$ 1.0
$\nu_1\text{PO}_4/\text{CH}_2\text{-wag}$	—	7.89 $\pm$ 0.64	6.45 $\pm$ 0.56*	7.67 $\pm$ 1.02
$\text{CO}_3/\nu_1\text{PO}_4$	—	0.155 $\pm$ 0.005	0.161 $\pm$ 0.007	0.155 $\pm$ 0.008
Crystallinity	FWHM ⁻¹	0.0460 $\pm$ 0.0006	0.0462 $\pm$ 0.0004	0.0457 $\pm$ 0.0005

*WT versus *Mmp2*^{-/-} or WT versus *Mmp9*^{-/-} was statistically significant as determined within each gender.

^a $\nu_1\text{PO}_4$ is the phosphate peak at 960 cm⁻¹; CH₂-wag is the peak related to the bending mode of the carbon-hydrogen at 1452 cm⁻¹; CO₃ is the carbonate peak at 1072 cm⁻¹.

among the genotypes ($p = .113$ from ANOVA). In addition, there were no differences in crystallinity among WT, *Mmp2*^{-/-}, and *Mmp9*^{-/-} mice ($p = .164$). Consistent with the decrease in tissue-level mineral-to-collagen ratio, nanoindentation modulus and hardness were less in the *Mmp2*^{-/-} tibial cortex than in the WT tibial cortex (Fig. 4). There were no differences in the nanoindentation properties with the loss of *Mmp9*^{-/-} (Fig. 4).

Discussion

This analysis of bones from *Mmp2*^{-/-} and *Mmp9*^{-/-} mice indicates that MMPs likely influence fracture resistance. In particular, they can affect trabecular architecture, cortical bone structure, and tissue composition. Moreover, our data suggest that MMPs with similar target substrates (eg, MMP-2 or gelatinase A and MMP-9 or gelatinase B) can have differential effects on the biomechanical properties of bone (Fig. 5). The loss of MMP-2 was found to weaken bone, and this effect was associated with a decrease in mineralization density of the tissue and an increase in porosity. In contrast, the loss of MMP-9 was found to not affect the bending strength of the whole femur despite causing a decrease in structure, but instead, MMP-9 deletion decreased bone toughness. The exact cause for this effect on the brittleness of bone is presently unknown, but the state of type 1 collagen of bone affects brittleness⁽³¹⁾ and MMP-9 may directly influence the organization extracellular matrix proteins found in bone.

A lower body mass could be associated with a reduction in the structural strength of femur midshaft via an indirect functional adaptation of the bone. Nonetheless, while the individual loss of

both MMP-2 and MMP-9 was associated with a reduction in body mass, the deletion of each gene did not have the same effect on peak force or structure of the femur midshaft (Table 2). The differential effects between the loss of MMP-2 and the loss of MMP-9 on the biomechanical properties of bone actually could be due to differences in which bone cells express primarily these two MMPs. MMP-9 is associated with osteoclast migration and angiogenesis.^(4,6,32-34) Specifically, *Mmp9*^{-/-} osteoclasts do not invade ossification centers adequately,⁽³⁴⁾ where they can remove calcified cartilage or expand the medullary canal, and this phenomenon could explain, in part, the observed differences in trabecular architecture and in cortical structure between the *Mmp9*^{-/-} and WT mice. That is, the dysfunction in osteoclast migration and angiogenesis could have affected the growth of the long bone in the *Mmp9*^{-/-} mice such that there was a reduction in endosteal and periosteal expansion (ie, lower I_{min}) and a decrease in the removal of trabeculae (ie, greater Conn.D). Differences in structure and architecture of the bone are less likely due to a disruption in osteoclast activity. Using a bone-resorption model of tracking ⁴⁵Ca release during a bone explant culture,⁽⁴⁾ Engsig and colleagues⁽³⁴⁾ observed no difference in the demineralization rate of the tibia between WT and *Mmp9*^{-/-} mice. In addition, histologic analysis of tartrate-resistant acid phosphatase (TRACP)-positive cells of bone sections from E17 to E19 embryos revealed similarities in number and size of osteoclasts.⁽³⁴⁾ Thus MMP-9 deficiency likely does not affect osteoclast activity (ie, matrix solubilization), with little histologic evidence that resorption is affected in *Mmp9*^{-/-} bones.^(6,34)

MMP-2, on the other hand, is expressed primarily by osteoblasts and plays a yet to be fully understood role in canaliculi formation.^(11,35) Specifically, *Mmp2*^{-/-} bones display a

Fig. 4. Nanoindentation-derived measurements (mean $\pm$ SD) of cortical bone tissue for WT ($n = 8$), *Mmp2*^{-/-} ($n = 11$), and *Mmp9*^{-/-} ($n = 9$) mice.

Characteristic of bone	Loss of MMP-2	Loss of MMP-9
Connectivity density of trabeculae	↓	↑
Mineralization density of trabeculae	↓	↓
Strength of vertebra	↓	—
Mineralization density of diaphysis	↓	—
Periosteal circumference	—	↓
Cortical porosity	↑	—
Strength of diaphysis	↓	—
Brittleness of diaphysis	—	↑
Tissue hardness	↓	—

Fig. 5. Summary of the effects of deleting MMP-2 and separately deleting MMP-9 on selected characteristics of bone. The dash indicates no change.

disrupted canalicular network but with little osteocyte death,^(11,35) and this phenomenon may impede the accumulation of mineral within the matrix. Thus this could explain the observed decrease in mineralization at the tissue level in *Mmp2*^{-/-} mice and, ultimately, the weakening of the *Mmp2*^{-/-} femur diaphysis and vertebra. The lack of a difference in the size of diaphysis ($I_{\min}$) and trabecular bone volume in the tibia metaphysis between WT and *Mmp2*^{-/-} mice reflects the reported observations of osteoblast function by Inoue and colleagues,⁽¹¹⁾ who found, for long bones, little difference in mineralization apposition rate and bone-formation rate per bone surface between the two genotypes. They also did not detect any appreciable differences in osteoblastic nodule formation or osteoclast formation in in vitro bone marrow cell culture assays.⁽¹¹⁾

To further understand how MMP-2 and MMP-9 are affecting the biomechanical properties of bone, the peak force endured by each bone was plotted against its moment of inertia, an index of structure, within each genotype (Fig. 6). $I_{\min}$ characterizes the distribution of bone tissue about the bending axis of the bone and can be directly proportional to stiffness, yield force, and peak force. This is the case for WT and *Mmp9*^{-/-} femurs, with a linear

regression fitting the force versus $I_{\min}$ data (Fig. 6). Interestingly, despite the fact that *Mmp9*^{-/-} femurs had a lower $I_{\min}$ than WT femurs, the *Mmp9*^{-/-} femurs were not any weaker than the WT femurs. Since μ CT and Raman spectroscopy did not reveal any differences in mineralization between these two genotypes, the loss of MMP-9 could be affecting collagen or another extracellular matrix protein in a way that strengthens the bone while also making it more brittle. Again, the integrity of collagen is a well-known determinant of bone toughness.^(36,37) As for *Mmp2*^{-/-} femurs, the lower strength likely was due to the lower Ct.TMD and higher Ct.Po of the *Mmp2*^{-/-} cortex compared with WT femurs (Table 2).

The compression strength of the L₅ vertebra depends on both trabecular and cortical compartments. There was a decrease in trabecular bone volume (BV/TV) and a decrease in Tb.TMD for the loss of both MMP-2 and MMP-9, but only *Mmp2*^{-/-} VBs were less stiff with lower peak and yield force than were the WT VBs. This suggests that other effects of MMP-9 on bone could be influencing biomechanical properties of the L₅ VB. Besides collagen, possible factors include an increase in Conn.D of the VB trabeculae and an increase in the thickness of the *Mmp9*^{-/-} VB cortex.

MMPs have been implicated in a loss of fracture resistance among humans. Mutations in the gene coding for MMP-2 are the likely cause of multicentric osteolysis, or “vanishing bone” disease, which is associated with fractures.⁽³⁸⁾ Polymorphisms of the *MMP9* gene in Japanese men have been associated with areal BMD, a correlate of bone strength,⁽³⁹⁾ and osteoporotic bone tissue was reported to have greater *MMP9* mRNA expression than normal tissue, with expression localizing to osteoclasts.⁽⁴⁰⁾ However, there are few investigations testing whether MMP inhibitors or agonists affect the fracture resistance of bone. In one study, minocycline, a tetracycline antibiotic that broadly inhibits collagenase activity, prevented the decrease in BMD following ovariectomy in aged rats,⁽⁴¹⁾ and this was associated subsequently with an increased expression of type 1 collagen and decreased expression of interleukin 6.⁽⁴²⁾ Minocycline also was reported to correct the decreases in bone formation that occur when rats are administered streptozotocin, which is a

Fig. 6. The expected direct relationship between peak force and $I_{\min}$ existed only for the WT and *Mmp9*^{-/-} femurs.

model of type 1 diabetes.⁽⁴³⁾ Nonetheless, the mechanism(s) responsible for these effects were not established, and moreover, these previous studies did not investigate the effect of inhibiting MMPs on the biomechanical properties of bone. Based on our reported differences in the biomechanical properties of bone between the deletion of MMP-2 and that of MMP-9, a broad-spectrum inhibition of collagenase activity likely would cause disparate changes to bone architecture, structure, and potentially composition.

Osteoblast-specific and osteoclast-specific knockouts of MMPs or selective MMP inhibitors may reveal the underlying mechanism of how proteolytic enzymes influence the fracture properties of bone. Indirect effects on bone quality are presently unknown. As mentioned previously, MMP-2 can activate TGF- β 1,⁽⁴⁴⁾ and a decrease in TGF- β activity increases tissue mineralization and modulus,^(15,16) but the TMD of *Mmp2*^{-/-} bones was not greater than that of WT bones—in fact, the opposite. In the context of TGF- β 1 being a coupling factor between bone resorption and formation,⁽⁴⁵⁾ there is the possibility that MMP-2 deficiency is affecting the recruitment of osteoblasts to sites of resorption, thereby giving rise to porosity near the endosteum (Fig. 3). Regardless of the exact mechanism, this study confirms previous reports that germ-line deletion of MMP-2 and separately MMP-9 causes reduced BMD^(11,35) and reduced bone length,⁽⁶⁾ respectively; and it further extends these changes to differential effects on the biomechanical, architectural, and compositional properties of bone. Of course, these effects could arise from the absence of these MMPs in chondrocytes during endochondral bone formation, but the persistence of the phenotype at 16 weeks of age, when bone remodeling and modeling are active, suggests that these MMPs are important to the organization of bone matrix with sufficient quality.

To date, there is a paucity of information on regulators of bone quality. The matrix metalloproteinases are potential candidates given their unique role in processing the organic matrix of bone and their importance to the actions of bone cells. The results from this study indicate that two gelatinases, MMP-2 and MMP-9, are likely necessary to produce bone tissue that is sufficiently resistant to fracture. In particular, MMP-2 likely influences mineralization, thereby affecting bone strength. Its influence on mineralization may be related to a role in the formation of a well-connected canalicular system. MMP-9, on the other hand, likely influences the architecture of trabecular bone and the structure of cortical bone. As such, it probably has some influence on bone strength, but it appears to be regulating primarily bone toughness or the brittleness of bone. These differential effects of MMPs suggest that bone quality is influenced by the type of MMPs respectively expressed by osteoblasts and osteoclasts.

Disclosures

All the authors state that they have no conflicts of interest.

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