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Gender-related changes in three-dimensional microstructure of trabecular bone at the human proximal tibia with aging

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Summary. Despite increasing interest in age- and gender-related bone alterations, data on trabecular microstructure at the proximal tibia are scarce. The aim of this study was to identify trabecular microstructural change at the human proximal tibia with age and gender, using micro-computed tomography (micro-CT) and scanning electron microscopy (SEM). Fifty-six proximal tibias from 28 Japanese men and women (57-98 years of age) were used in this study. The subjects were chosen to give an even age and gender distribution. Both women and men were divided into three age groups, middle (57-68 years), old (72-82 years) and elderly (87-98 years) groups. The trabecular bone specimens from the medial compartment of the proximal tibial metaphysis were examined. Trabecular bone mineral density (BMD), bone volume fraction (BV/TV) and trabecular thickness (Tb.Th) decreased between the middle-aged and elderly groups similarly in women and men. However, trabecular number (Tb.N) decreased by 13% between the middle-aged and elderly groups in women and nearly double that in men. As compared with women, men had higher BV/TV and lower trabecular separation (Tb.Sp) in the old age and elderly groups, and higher Tb.N and connectivity density (Conn.D) in the elderly group. Increased trabecular resorbing surfaces, perforated or disconnected trabeculae and microcallus formations were observed with age. These findings indicate that both BMD and BV/TV decreased at the proximal tibia with age similarly for women and men, but significant differences between women and men were observed for some microstructural parameters. These findings illustrate potential mechanisms underlying osteoporotic proximal tibial fracture.

Key words: Proximal tibia, Trabecular bone, Three-dimensional microstructure, Micro-CT, Scanning electron microscopy

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Introduction

Osteoporosis is an age-related skeletal disease that is characterized by low bone mass and deteriorated bone quality. It decreases bone strength and increases susceptibility to fracture (National Institutes of Health, 2000). Bone quality is a key component for assessing fracture risk and is determined by complex features such as bone mineral density (BMD), bone mechanical properties and bone microstructure. As the changes in trabecular bone microstructure may impact bone strength independently of bone mass, there is increasing interest in assessing bone microstructure, with the ultimate goal of improving the prediction of fracture risk. It is clear that trabecular bone is continually altered with aging, mechanical loading and pathology against a specific genetic background. The analysis of local trabecular bone architecture is essential in obtaining a fuller appreciation of the contribution of trabecular structure to mechanical competence, as well as its pathophysiology (Stauber et al., 2006). Analyses of trabecular microstructure have been mainly performed in regions most susceptible to fractures, such as spine, proximal femur and radius (Riggs et al., 2004; Khosla et al., 2006; Eckstein et al., 2007; McDonnell et al., 2007; Chen et al., 2008a, 2010; Lochmüller et al., 2008). Studies of the proximal tibia also have an important clinical significance, as it is fractured in aging patients, specifically those suffering from osteoporosis (Krieg,

2003). The proximal tibia, with its rich trabecular network, can be used as a donor site for bone grafting (Walker et al., 2009) and it is the most easily accessible site for quantification of BMD and bone microstructure. The trabecular bone properties of the proximal tibia have been previously examined using different techniques (Ding et al., 1997, 2002; Ding and Hvid, 2000; Khodadadyan-Klostermann et al., 2004; Thomsen et al., 2005; Hakulinen et al., 2006; Lancianese et al., 2008). Histomorphometric analysis showed that the degree of anisotropy (DA) increased significantly with age (Ding et al., 2002). Age-related changes for trabecular thickness (Tb.Th) and structure model index (SMI) became significant after 80 years of age (Ding and Hvid, 2000). The trabecular BMD of the proximal tibia was reported to be decreased with age in females over the age of 60 years (Khodadadyan-Klostermann et al. 2004). To our knowledge, nevertheless, no specific reports concerning gender-related variations in trabecular microstructure at the proximal tibia with aging are available.

Through the advancement in spatial resolution of 3D imaging systems and computational methods, micro-CT has become a frequently used method for unbiased quantities of trabecular microstructural parameters (Hildebrand and Rüegsegger, 1997; Hildebrand et al., 1999). With this technique, studies have been conducted to measure the changes of 3D microstructural parameters with age. Micro-CT provides a tool for obtaining both 2D histomorphometric data and measurement of 3D connectivity. However, the detailed morphology of trabecular bone surfaces cannot be fully identified by this method. Scanning electron microscopy (SEM) has proven effective in investigating the activity states of bone surfaces (Boyde, 2003; Banse et al., 2005). The aim of the present study was therefore to identify trabecular 3D microstructural changes at the proximal tibia with age and gender, using micro-CT and SEM. It was hypothesized that women had less trabecular bone mass with age than men.

Materials and methods

Fifty-six proximal tibiae from 28 Japanese men (age range 57-98 years; mean age 77.6±12.8 years) and women (age range 57-98 years; mean age 77.4±13.0 years) were obtained during dissection in a gross anatomy course. In this study, the written informed consent was obtained for all subjects to offer cadavers for anatomical education and research. The subjects were chosen to give an even age and gender distribution. Both women and men were divided into three age groups: middle (age range 57-68 years; N=9), old (age range 72-82 years; N=9), and elderly groups (age range 87-98 years; N=10). The cause of death was known for each individual, with no medical history of metabolic bone disease or cancer. The causes of death included cardiac failure, cerebral hemorrhage, pneumonia, multisystem organ failure, liver failure, and natural death. No other medical history was available from these donors. No detectable visible or radiological signs of disease or osteoarthritis were present in any of the specimens used.

The proximal tibia was cut in order to obtain trabecular specimens of 8x8x8 mm cube using a low speed diamond saw (Isomet, Buehler). The medial compartment of the proximal tibial metaphysis was chosen rather than the lateral side as it has been shown that 75% of the load across the knee is carried by the medial compartment (Hsu et al., 1990), and it is therefore reasonable to assume that the medial compartment is more sensitive to skeletal unloading. Furthermore, the trabecular structure at the lateral compartment of the proximal tibial metaphysis is less well defined, owing to the particular biomechanics of the tibiofibular articulation (Thomsen et al., 2005). The specimens were cut through the midpoint of medial tibial plateaus cranially, along the longitudinal axis of tibia shaft, corresponding to the direction of physiological loading (Fig. 1). All specimens were taken 6 mm beneath the subchondral bone plate, as the normalization of forces appears to be nearly complete after approximately 6 mm of depth from the articular cartilage (Patel et al., 2003).

Trabecular bone microstructure was analyzed by using cone-beam X-ray micro-CT system (MCT-CB100MF, Hitachi Medical Corporation, Kashiwa, Japan) as described previously (Chen et al., 2008a,b,

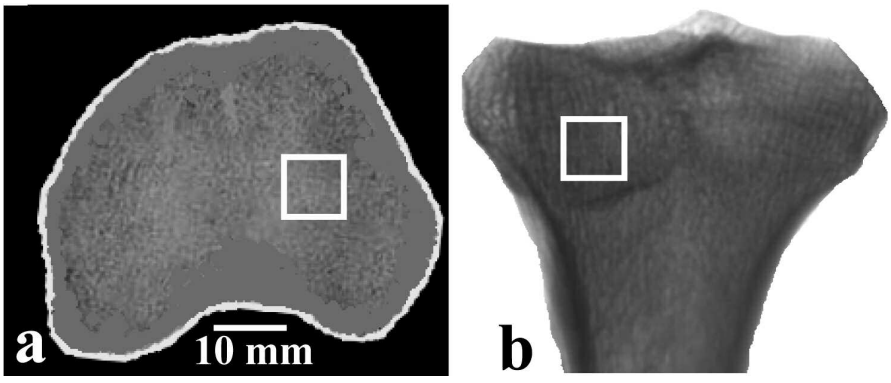

Fig. 1. Stereomicroscopic images of the transverse **(a)** and frontal **(b)** sections from the proximal tibia. The medial region (square) of the proximal tibia for micro-CT scanning is shown.

2009, 2010). Trabecular bone was scanned continuously with increments of 15 μm thickness for 512 slices with a tube voltage of 50 kV, tube current of 0.1 mA. The voxel size was 15x15x15 μm. After scanning, the micro-CT image data was transferred to a work station, and microstructural parameters were calculated using 3D trabecular bone analysis software TRI/3D-BON (Ratoc System Engineering Co. Ltd., Tokyo, Japan). TRI/3D-BON builds 3D models from serial tomographic datasets for visualization and morphometric analysis as described (Chen et al., 2008a,b, 2009, 2010). The 3D images were segmented into voxels identified as bone and marrow. The noise was reduced by median filter processing with a mask size of 3x3 pixels. A uniform threshold value was used to segment the images for all specimens. The isolated small particles in marrow space and the isolated small holes in bone below 10 pixels were removed using a cluster-labeling algorithm (Odgaard and Gundersen, 1993). Subsequently, the indices of trabecular bone structure were calculated. Bone volume (BV) was calculated using tetrahedrons corresponding to the enclosed volume of the triangulated surface (Lorensen and Cline, 1987). Trabecular BV/TV was calculated with bone volume (BV) and total tissue volume (TV). Trabecular thickness (Tb.Th), trabecular separation (Tb.Sp) and trabecular number (Tb.N) were based on direct measures by a distance transformation method (Hildebrand and Rüegsegger, 1997). The structure model index (SMI) is a parameter that quantified the characteristic form of a 3D described structure in terms of the plate-like or rod-like nature of the complete structure (Hildebrand and Rüegsegger, 1997). Connectivity density (Conn.D) is a topological parameter that estimates the number of trabecular connections per cubic millimeter (Odgaard, 1997). The degree of anisotropy (DA) defines the direction and magnitude of the preferred orientation of trabeculae and uses the ratio between the maximum and minimum radii of the mean intercept length ellipsoid (Harrigan and Mann, 1984). All the above parameters were computed in 3D without model assumptions required for 2D-based analysis (Hildebrand et al., 1999).

Trabecular BMD measurements were performed using micro-CT system as described previously (Chen et al., 2008b, 2009). To calibrate CT units to equivalent bone mineral concentration, all bone samples were scanned by micro-CT system together with a calibration phantom (Kyoto Kagaku Co. Ltd., Kyoto, Japan). Trabecular BMD was calculated using software TRI/3D-BON-BMD (Ratoc System Engineering Co. Ltd., Tokyo, Japan).

After micro-CT scanning, trabecular bone specimens were cleaned with a fine jet of water to remove bone marrow. They were then treated with 5% sodium hypochlorite solution to remove residual marrow. The specimens were dehydrated through ascending concentrations of acetone and critical-point dried, mounted on stubs and coated with gold/palladium using an ion sputter. Trabeculae were examined with a scanning electron microscope (Hitachi S-3500 N SEM). Twenty micrographs at a final magnification of 200x were taken from each specimen randomly. The trabecular resorbing surfaces, showing depressions or pits with bright scalloped edges, indicative of osteoclastic resorptions, have been described extensively (Jayasinghe et al., 1993; Mosekilde, 1993; Chen et al., 2004, 2008a). In the present study, the percentage area occupied by the resorbing surface was estimated as described previously (Chen et al., 2004, 2008a).

All data were presented as mean ± SD. Statistical analysis was done using SPSS version 14 (Chicago, IL, USA). Correlation between BV/TV and age, between BMD and age for both women and men was studied by linear regression analysis. One-way analysis of variance (ANOVA) was used to compare trabecular microstructural parameters and BMD in the three age groups. If the F-test showed significance, Fisher's protected least squares differences test was used for *post hoc* multiple comparisons. Statistical analysis of differences between women and men of the same age group was made by Student's t-test. The same statistical analysis was performed for the percentage area of the trabecular resorbing surface. Differences were considered significant at $p<0.05$.

Results

Table 1 shows the mean values of microstructural

Table 1. Trabecular microstructural parameters of the proximal tibia in three age groups of women and men.

	Middle (57-68 years)		Old (72-82 years)		Elderly (87-98 years)	
	Women (N=9)	Men (N=9)	Women (N=9)	Men (N=9)	Women (N=10)	Men (N=10)
BV/TV (%)	22.5±3.1	24.2±2.8	19.9±2.7[b]	22.1±3.0[a,b]	17.8±2.4[b,c]	19.6±2.7[a,b,c]
Tb.Th (μm)	179.6±21.8	188.4±20.4	172.1±19.1	178.4±19.0	161.5±18.2[b]	169.0±18.9[b]
Tb.N (/mm)	1.34±0.15	1.37±0.17	1.25±0.14	1.32±0.14	1.17±0.13[b]	1.28±0.14[a]
Tb.Sp (μm)	635.4±73.4	580.5±76.0	682.2±81.6	614.8±76.3[a]	706.9±82.5[b]	638.0±77.1[a,b]
SMI	1.09±0.25	1.06±0.23	1.18±25[b]	1.15±0.24[b]	1.30±0.24[b]	1.25±0.20[b]
Conn.D (/mm^3)	3.67±0.42	3.89±0.41	3.44±0.34	3.70±0.36	3.16±0.26[b,c]	3.49±0.31[a,b]
DA	1.75±0.18	1.71±0.17	1.68±0.20	1.74±0.21	1.63±0.19	1.69±0.19

Values are mean ± SD. [a]: $p<0.05$, between women and men of the same age group. [b]: $p<0.05$, vs. middle-aged group. [c]: $p<0.05$, vs. old age group.

parameters in three age groups of women and men. As is evident, the middle-aged group had the highest values of BV/TV, Tb.Th, Tb.N, and Conn.D and the lowest values of Tb.Sp and SMI for both women and men. In women, BV/TV decreased by 12% and 11% between the middle-aged and old age groups, and between old age and elderly groups, respectively. Tb.Th and Tb.N decreased by 10% and 13% between the middle-aged and elderly groups, respectively (Table 1). Consistently, Tb.Sp increased with age. SMI increased by 19% between the middle-aged and elderly groups, indicating a shift toward a more rod-like structure in the elderly group. The middle-aged group had the highest value of Conn.D, which decreased by 18% between the middle-aged and elderly groups in women. Age-related changes of BV/TV and Tb.Th showed similarity in women and men among the three age groups. However, in women, Tb.N decreased by 13% between the middle-aged and elderly groups and nearly doubled that in men. As compared with women, BV/TV was significantly higher and Tb.Sp was significantly lower in men of the old age and elderly groups. Tb.N and Conn.D were significantly higher in men of the elderly group. There were no significant differences for DA between women and men among the three age groups. Trabecular BMD declined by 14% and 11% between the middle-aged and elderly groups for women and men, respectively (Table 2). Compared with women, BMD had a tendency to be higher in men. The linear regression analysis showed that both BV/TV and BMD decreased significantly with age for both women and men (Fig. 2). From 57 to 98 years of age, BV/TV decreased by 7% and 6% per decade for women and men, respectively, while BMD declined by around 4% per decade (Tables 1, 2).

Figure 3 shows the typical 3D reconstructions of trabecular bone of the middle-aged and elderly groups for both women and men. Images of the sample with BV/TV that were closest to the mean BV/TV were reconstructed in each group. The trabecular bone volume fraction was highest in men of the middle-aged group and lowest in women of the elderly group. Women displayed lower values of BV/TV than men.

The SEM images of trabecular bones at the proximal tibia from women were shown in Fig. 4. The characteristics of the resorbing surfaces (R) showed considerable variability at different ages both for women and men. These variations were related to the trabecular

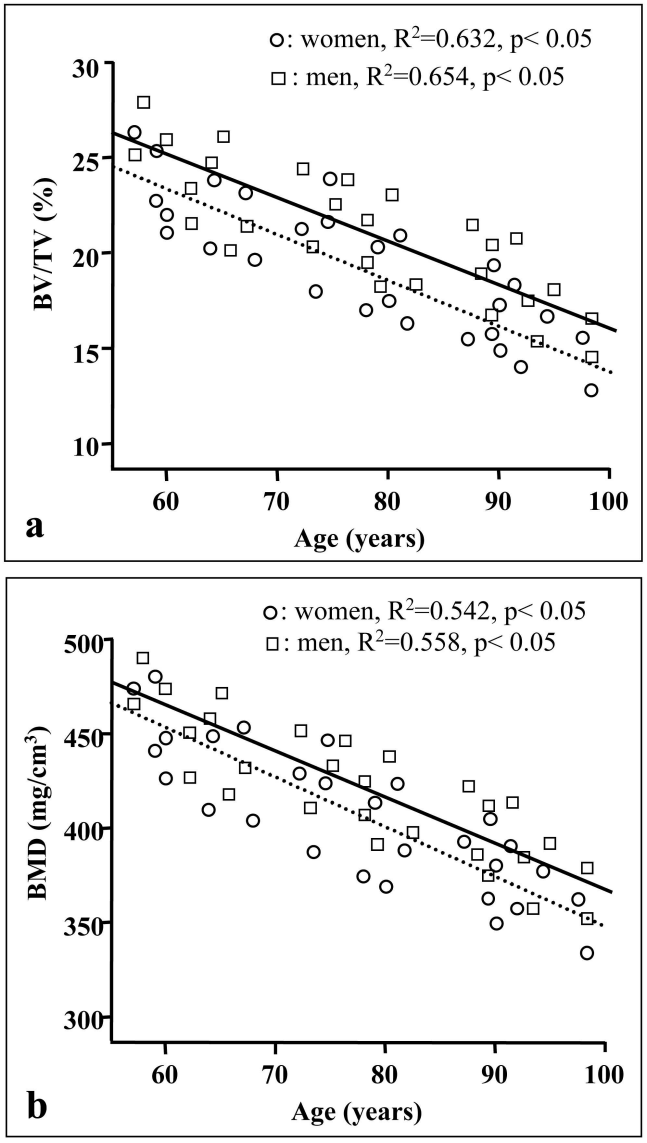

Fig. 2. Linear relationship between BV/TV and age **(a)**, between BMD and age **(b).** Age-related decreases in BV/TV and BMD showed similarly in women and men.

Table 2. Trabecular bone mineral density (BMD) and percentage area of trabecular resorbing surface at the proximal tibia of three age groups of women and men.

	Middle (57-68 years)		Old (72-82 years)		Elderly (87-98 years)	
	Women (N=9)	Men (N=9)	Women (N=9)	Men (N=9)	Women (N=9)	Men (N=9)
BMD (mg/cm^3)	435.4±45.6	451.6±47.8	406.2±44.4	428.5±47.6	372.7±43.7^{b}	403.2±42.4^{b}
Resorbing surface (%)	4.25±0.53	4.02±0.46	4.87±0.58^{b}	4.46±0.55^{a,b}	5.49±0.67^{b,c}	4.92±0.64^{a,b,c}

Values are mean ± SD. [a]: $p<0.05$, between women and men of the same age group. [b]: $p<0.05$, vs. middle-aged group. [c]: $p<0.05$, vs. old age group.

structural alternations, such as thinning, perforation and disruption of the trabeculae that occur with age. The size and shape of the resorbing surfaces varied from long, shallow (Fig. 4c), elongated furrows to rounded deep lacunae (Fig. 4a,b). The amount of bone removed by the resorption varied from superficial skimming of surfaces to complete perforation or disconnection of the trabeculae (Fig. 4c). In some regions, the trabecular microcallus formation was observed (Fig. 4d).

The percentage area of trabecular resorbing surface is shown in Table 2. The percentage area of trabecular resorbing surface had a tendency to increase with age for

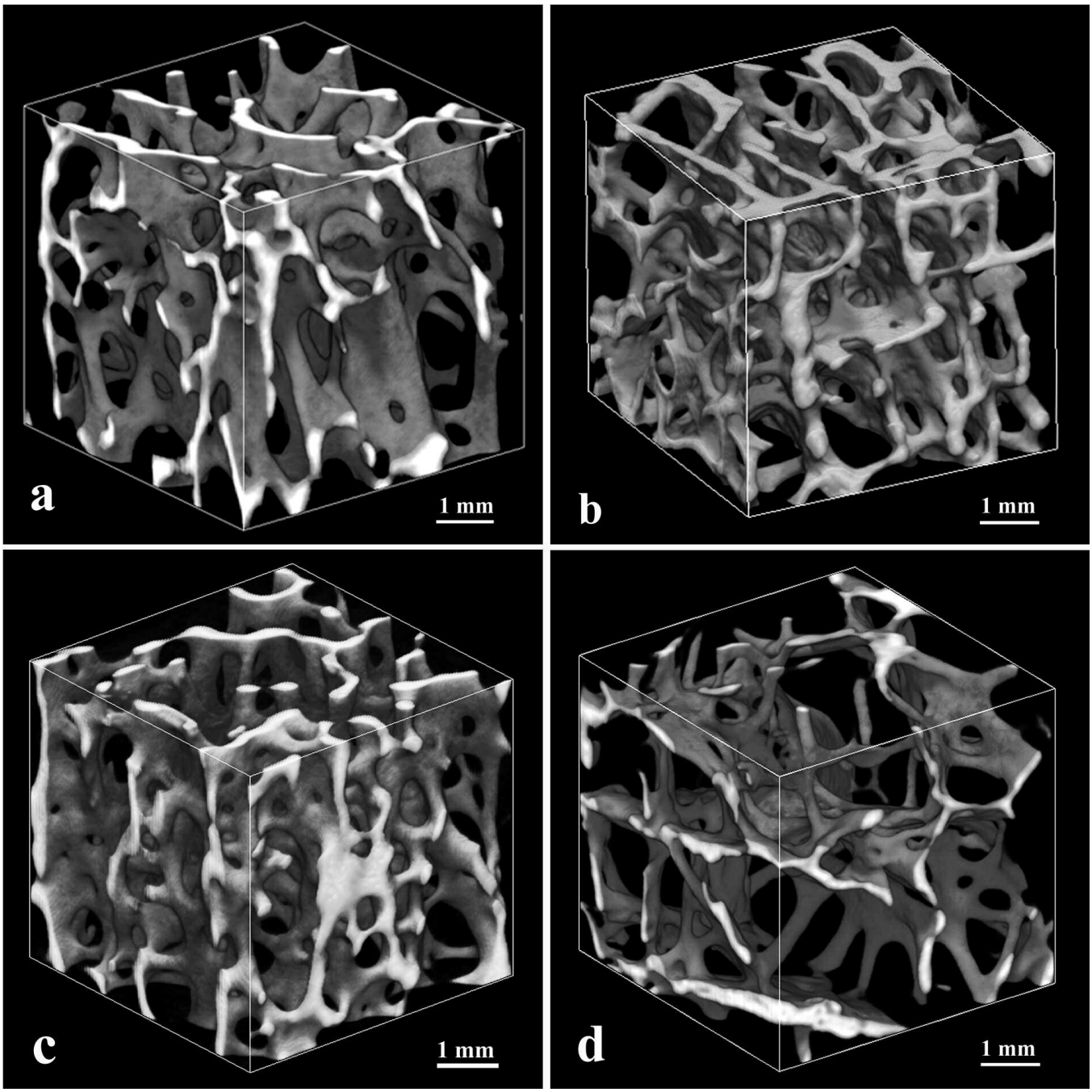

Fig. 3. Three-dimensional reconstructed images of trabecular microstructure at proximal tibia from a man aged 62 years **(a)**, a man aged 92 years **(b)**, a woman aged 62 years **(c)**, and a woman aged 92 years **(d)**. The trabecular bone volume fraction is highest in man aged 62 years and lowest in woman aged 92 years.

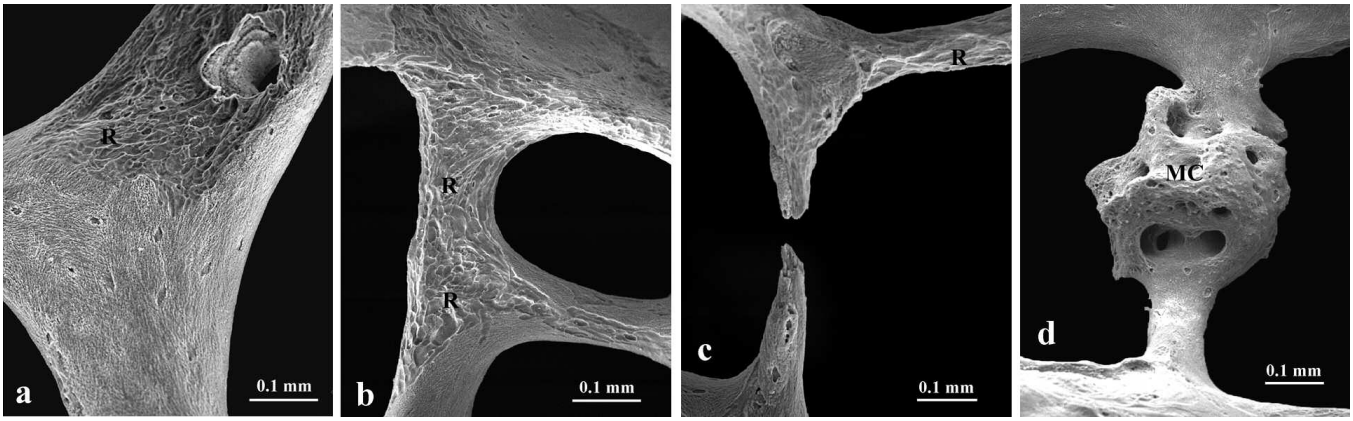

Fig. 4. SEM photographs of proximal tibial trabeculae from women aged 72 **(a)**, 88 **(b)**, 96 **(c),** and 76 years **(d)**. The rounded deep resorption lacunae **(a, b)**, elongated shallow resorption furrows **(c)** and trabecular microcallus formation **(d)** are observed. R: resorbing surfaces; MC: microcallus.

both women and men. Compared with the middle-aged group, the percentage area of trabecular resorbing surface was significantly higher in old age and elderly groups. Compared with women, the percentage area of trabecular resorbing surface was significantly lower in men of the old age and elderly groups (Table 2).

Discussion

In this study, the trabecular bone microstructure of the human proximal tibia with age and gender was examined. It is well accepted that age-related bone loss is an important factor leading to decreased bone strength, enhanced bone fragility and fracture risk in the elderly. It has been shown that, on average, about 70% of the variation in bone strength is determined by BMD (Ammann and Rizzoli, 2003). The correlation between BMD and trabecular bone strength of the proximal tibia was highly significant (Petersen et al., 1996). In the present study, it was demonstrated that, from 57 to 98 years of age, trabecular BMD declined by around 4% per decade. The decline rate was similar for women and men at this age. However, women had consistently lower BMD measurements than men of the same age. Possible explanations for the gender difference in BMD are that women reach a lower peak bone mass before they start losing bone, or that any accelerated loss occurs earlier, perhaps, as has been suggested, around the menopause (Seeman, 2002). It is likely that much of the female preponderance for fractures is related to the lower bone mass of women compared with men.

In addition to BMD, bone microstructure is an important determinant of bone strength (McDonnell et al., 2007). The trabecular microstructure can be evaluated by quantifying histomorphometric parameters, such as BV/TV, Tb.Th, Tb.N, SMI and Conn.D. Age-related trabecular bone loss at the proximal tibia included a decrease in BV/TV, Tb.Th, and Conn.D, an increase in Tb.Sp and SMI (Ding and Hvid, 2000; Ding et al., 2002). The results of the present study are in line with these findings. There is considerable controversy regarding the microstructural basis for age-related decrease in trabecular bone (Parfitt, 1984; Ding and Hvid, 2000; Eckstein et al., 2004; Khosla et al., 2006; Stauber and Müller, 2006; Chen et al., 2008a, 2010). In the present study, it was found that the decline in BV/TV and Tb.Th with age was similar for women and men. However, the decrease in Tb.N for women was nearly twice as much as men. It was considered that age-related bone loss at the proximal tibia in women was caused by decreases in both Tb.N and Tb.Th, whereas in men, the primary mechanism for the decrease in BV/TV was trabecular thinning. Based on finite element modeling, reductions in Tb.N had a 2- to 5-fold greater impact on bone strength compared with reductions in Tb.Th that resulted in similar decreases in bone volume (Silver and Gibson, 1997).

Trabecular bone has a complex 3D structure that consists of interconnecting plates and rods. An estimation of plate or rod characteristic of trabeculae can be determined by measuring SMI. This is an important structural feature, which impacts strongly on the mechanical properties of the trabeculae. Ding et al. (2002) analyzed age-related changes in proximal tibial trabeculae from 40 normal donors aged 16 to 85 years. It was found that SMI was higher in the old age group. In the present study, SMI increased between the middle-aged and elderly groups by 18-19% for women and men. A shift toward a more rod-like structure with age was observed and hence is likely to be more susceptible to bending and buckling failure modes. Conn.D is a fundamental property of 3D trabecular network and is crucial in the maintenance of bone strength. As BV/TV decreases, there is a corresponding decrease in Conn.D, possibly due to the loss of small interconnecting trabeculae with small initial diameter (Chen et al., 2008a, 2010). The present study showed that Conn.D significantly decreased with age. Similar results were

reported previously (Ding et al., 2002; Chen et al., 2008a, 2010). However, several questions concerning the changes of trabecular connectivity under pathological condition remain unanswered.

SEM is a powerful technique for investigating trabecular bone microstructure. By using SEM, it is possible to distinguish trabecular resorption styles, which are important for the determination of bone architectural integrity and bone quality (Jayasinghe et al., 1993; Mosekilde, 1993; Boyde, 2003; Banse et al., 2005). The present results showed that the percentage area of trabecular resorbing surface was significantly increased with age. Some trabeculae were completely perforated or disconnected. Age-related trabecular bone loss at the proximal tibia is caused by perforation and thinning of trabeculae. In this study, Several trabecular microcallus formations on the thin trabeculae were observed. Microcallus is described as a small mass of woven bone, which was often seen at the vertebra, mainly on vertical thin trabeculae. Microcallus can be seen as an attempt to preserve or repair a trabecula (Hahn et al., 1995; Cheng et al., 1997; Banse et al., 2005; Chen et al., 2008a). However, what triggers the microcallus formation is still subject to debate, and the presented images do not clarify the situation.

The presented methodology differs from previous studies in that the bone density and bone microstructure were examined with micro-CT and SEM. Both methods allow direct assessment of the 3D structures. To obtain microstructural parameters using micro-CT and SEM, the specimen size is usually limited. In the present study, the trabecular specimens of 8x8x8 mm were used for microstructural analysis. This can only provide data for relatively small localized bone volume. The bone microstructural properties are known to depend strongly on the spatial resolution, which is inversely related to the specimen size. The specimen size of this study is similar to that of the previous studies for human proximal tibia. Great care was taken to harvest specimens at exactly the same anatomical location in all subjects. Another limitation was the relatively small number of cases studied, so the power of this study to demonstrate statistically significant differences is limited. All specimens of this study were obtained from subjects aged 57 years or older. With more cases from young individuals, it might be possible to offer a more accurate characterization of the proximal tibia's microstructure and how it varies with age and gender. Though the cause of death was known for all individuals, the complete medical histories were not available for the cadaver donors studied. This could include subjects who may have had medical conditions and lifestyle habits that could affect bone metabolism. As all individuals of this study were Japanese, the results represent age-related microstructural changes in a presumably Japanese population, in which lifetime loading patterns may differ from Western women and men due to differences in physical activity, bone geometry and other factors. Also, there might be secular effects in which the oldest subjects may differ from the younger ones due to different conditions while achieving peak bone mass.

In conclusion, the present study demonstrated that from 57 to 98 years of age, the trabecular BV/TV at the proximal tibia decreased by 7% and 6% per decade for women and men, respectively. Trabecular BMD declined around 4% per decade for both women and men. Men had relatively higher BV/TV than women. These findings reiterate the hypothesis that women had less trabecular bone mass with age than men. In women, age-related trabecular bone loss is associated with decreases in both Tb.N and Tb.Th, whereas in men, trabecular thinning is relatively greater than trabecular loss. Compared with women, men had higher BV/TV and lower Tb.Sp in the old age and elderly groups, and higher Tb.N and Conn.D in the elderly group. The microstructural basis for age-related bone loss at the proximal tibia varied between women and men. The present findings may serve as reference for ethnic comparison with age and gender, and may help to provide more insight into proximal tibia fracture risk.

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