Warfarin-induced impairment of cortical bone material quality and compensatory adaptation of cortical bone structure to mechanical stimuli

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Abstract

Long-term warfarin use has been reported to increase fracture risk of rib and vertebra but not hip in elderly patients, but the mechanisms remain unknown. We hypothesized that warfarin would impair bone material quality but could not weaken bone strength under conditions with higher mechanical stimuli. To test this hypothesis, rats were randomized to vehicle or warfarin group at 4 weeks of age and subsequently weight matched into a sedentary or jumping exercise group at 12 weeks of age. At 6 months of age, osteocalcin content, bone mineral density (BMD), mineral size, material properties, morphological parameters, and biomechanical properties of cortical bones were evaluated. In order to seek evidence for a common mechanism of action, effects of nucleation rate of mineral crystals on their rigidity were also investigated using computer simulation. In humeral cortical bones, warfarin did not change BMD, but markedly decreased osteocalcin content, diminished mineral size, and impaired material hardness. Consistent with these results, our computer-simulation model showed that osteocalcin-induced delay of mineral crystal nucleation decreased mineral formation rate, increased mean and distribution of mineral sizes, and strengthened mineral rigidity. In tibial cortical bones, warfarin decreased material ultimate stress; however, under jumping exercise, warfarin increased cross-sectional total and bone areas of these tibiae and completely maintained their biomechanical properties including work to failure. Collectively, our findings suggest that long-term warfarin therapy weakens rib and vertebra by impairing cortical bone material quality due to a marked decrease in osteocalcin content but could not reduce hip strength through compensatory adaptation of cortical bone structure to higher mechanical stimuli.


Introduction

Accumulating evidence has shown that there are various risk factors for osteoporosis which are independent of areal bone mineral density (BMD) measured by dual-energy X-ray absorptiometry (Kanis 2002, Poole & Compston 2006). Indeed, osteoporotic fracture is closely related to skeletal fragility that is determined by bone material and structural quality as well as its quantity (Seeman & Delmas 2006). For example, osteocalcin, the most abundant non-collagenous protein in bone, is incorporated into bone through carboxylation (Szulc et al. 1993, 1996, Vergnaud et al. 1997, Luukinen et al. 2000, WHO Scientific Group 2003), independently of areal BMD (Vergnaud et al. 1997, WHO Scientific Group 2003). In fact, carboxylation of osteocalcin is promoted by vitamin K, and high-dose vitamin K2 (menaquinone-4) therapy reduces fracture incidence without increasing areal BMD in older patients with osteoporosis (Cockayne et al. 2006, Iwamoto et al. 2006, Sugiyama 2007).

Consistent with these lines of evidence, warfarin, a vitamin K antagonist, has been suggested to increase fracture risk by impairing bone quality (Eastell 2006); warfarin does not significantly affect lumbar or hip areal BMD (Caraballo et al. 1999a), while its long-term use has been reported to increase fracture risk of rib and vertebra in elderly patients (Caraballo et al. 1999b, Gage et al. 2006). Thus, warfarin appears to induce skeletal fragility through a decrease in bone osteocalcin content. However, long-term therapy with warfarin has been reported not to increase hip fracture risk in the elderly (Jamal et al. 1998, Mamdani et al. 2003, Gage et al. 2006). Currently, although warfarin is prescribed to
millions of people worldwide to decrease their risk of clotting (Hirsh et al. 2003), the mechanisms by which this drug affects the skeleton have been poorly understood.

The skeleton adapts to its mechanical circumstances and bone strain generated by mechanical loading is an essential factor to determine bone structure and strength (Ehrlich & Lanyon 2002). For example, mechanical load-induced expansion of cortical bone structure effectively restricts skeletal fragility; however, such a structural change cannot be detected properly as areal BMD. Considering that femoral neck areal BMD tends to be higher when compared with lumbar areal BMD in patients treated with warfarin (Caraballo et al. 1999a) and the degree of bone strain from mechanical loads in ambulant people is much higher at the hip than at the rib or vertebra, we previously hypothesized that 1) long-term warfarin administration would impair bone material quality due to a decrease in osteocalcin content, but 2) the impaired bone material quality could not weaken the skeleton at sites subjected to higher mechanical stimuli, such as the hip, because the impairment of bone material quality increases bone strain from mechanical loading (Sugiyama et al. 2002a).

The objective of the present study was to test these two hypotheses. We investigated the effects of long-term warfarin administration on cortical bones in adult rats with or without vertical jumping exercise. Based on the findings that osteocalcin mainly delays crystal nucleation without inhibiting crystal growth in mineral formation de novo (Hunter et al. 1996) and the rigidity of bone tissue is produced by tiny mineral crystals (Fratzl et al. 2004), we also analyzed the effects of nucleation rate and growth speed of mineral crystals on their rigidity in computer simulation, in order to seek evidence for a common mechanism of action. Here, we show several pieces of evidence which are clearly consistent with our hypotheses and provide reasonable explanations for the highly controversial findings concerning the effects of long-term warfarin use on fracture risk in older patients (Jamal et al. 1998, Caraballo et al. 1999b, Mamdani et al. 2003, Gage et al. 2006, Rejmark et al. 2007).

Materials and Methods

Animals and treatments

After 1 week of acclimation, 4-week-old female Fisher 344 rats (Charles River Laboratories Japan Inc., Yokohama, Japan) were randomly assigned to vehicle group (s.c. injection of distilled water for 3 alternate days/week (n = 30)) or warfarin group (s.c. injection of warfarin potassium (supplied from Eisai Co. Ltd, Tokyo, Japan) dissolved from distilled water for 3 alternate days/week (n = 30)). At 12 weeks of age, each group was weight matched into sedentary control group (n = 14 each group) or vertical jumping exercise group (20 jumps (40 cm height)/day, 3 alternate days/week (n = 14 each group)); four rats in the warfarin group were excluded because of slight and temporary bleeding from the nose and/or in the eyes and two rats in the vehicle group were excluded because of the weight matched to the warfarin group. Vertical jumping exercise was performed using a method reported previously (Umemura et al. 1997). All rats were group-housed (n = 4–5) in sterilized cages under standard laboratory conditions and fed a synthetic diet containing 0-5% calcium, 0-66% phosphate, and 0-2 mg/100 g vitamin K (CLEA Japan Inc., Tokyo, Japan) until euthanasia. Analysis of fresh bones was performed carefully from 24 to 27 weeks of age (four rats (one rat per group) per day). Experimental procedures were reviewed by the committee for ethics in animal experiments at Yamaguchi University School of Medicine. They were carried out under the control of the guidelines for animal experiments at Yamaguchi University School of Medicine and the law (No. 105) and notification (No. 6) of the Japanese government.

Measurement of bone osteocalcin content

Fresh left humeral mid-shaft cortical bones (10 mm length in a longitudinal direction) were immediately stored at −80 °C after removal of soft tissue and bone marrow. Osteocalcin content in all bone samples was measured at the same time as described previously (Hara et al. 2005).

Measurement of bone leaky surface acoustic wave velocity

Fresh cortical bones (10 mm length in a longitudinal direction) cut from right humeral mid-shaft ventromedial plane region were ground and then polished using 0-035 μm grit colloidal silica; the final size of these specimens was ~10 mm length, 2 mm width, and 1 mm thickness, and the polished surfaces of the cortical bones were almost midway between the periosteum and the endosteum. Leaky surface acoustic wave velocity in the polished surfaces was measured using a scanning acoustic microscope (Hitachi Kenki Fine Tech Co. Ltd, Tokyo, Japan). Data were collected as a mean of two distinct measurements (1 mm length in a longitudinal direction, 100 MHz at 29 °C).

Analysis of bone material hardness and elastic modulus

After measurements of leaky surface acoustic wave velocity, hardness and elastic modulus in the transverse direction, on the same polished surfaces, were determined by nanoindentation using a three-sided Berkovich tip (Elionix Co. Ltd, Tokyo, Japan). Data were collected as a mean of ten distinct indents (a target force of 1 mN at a constant loading rate of 100 μN/s at 29 °C).

Curve-fit analysis of the ν1, ν3 phosphate region of hydroxyapatite spectra

After nanoindentation, the analyzed surfaces were polished again using 0-035 μm grit colloidal silica to remove the
Measurements of BMD and bone morphological parameters

After Fourier transform infrared microspectroscopy, BMD of the specimens was measured by peripheral quantitative computed tomography (Stratec Medizintechnik GmbH, Pforzheim, Germany). BMD and cross-sectional bone morphological parameters of tibial mid-shaft cortical bones were also measured using peripheral quantitative computed tomography and the parallel-axis theorem (Turner & Burr 2001).

Analysis of biomechanical and intrinsic material properties of bone

Biomechanical properties (ultimate load, stiffness, and work to failure) of fresh tibial mid-shaft cortical bones were determined by three-point bending (Tokyo Testing Machine, Toyohashi, Japan; 10 mm loading span at a cross-head speed of 2 mm/min at 37 °C). Intrinsic material properties (ultimate stress, Young’s modulus, and toughness) were evaluated as described previously (Turner & Burr 2001). Tibial length was measured with Vernier calipers.

Conceptual computer-simulation analysis of mineral crystal formation

Within a closed circular cylindrical space of the diameter $D$ and the length $L = 2D$, crystal nuclei of the specified number $N$ are assumed to be distributed with the specified time interval $T$, at arbitrary coordinate $(x_n, y_n, z_n)$ $(n = 1, 2, 3, \ldots, N)$ determined by the pseudo-random number (Matsumoto & Nishimura 1998), where $n$ is the discernible number of crystals. The scale is $D < 1 \mu m$. Since the precise three-dimensional nanostructure of bone mineral crystals remains unclear (Fratzl et al. 2004), each crystal is assumed to have a circular cylindrical shape of diameter $d_n$ and length $l_n$, and is approximated by analytically defined six-dimensional components as

$$\text{Crystal}_n = f(x_n, y_n, z_n, d_n, l_n, t)$$

where $t$ is the progress time after arising. Considering that bone mineral crystals will align with their long axes parallel to the collagen fibril axis in bone (Fratzl et al. 2004), the longitudinal axis of each crystal is parallel to the $z$ axis of the closed space in the longitudinal direction. Each crystal nucleus has a diameter of $1/250D$ and a length of $1/250D$ at first, and grows at the same specified velocity $V$ in the transverse and the longitudinal directions. Since the transverse growth is strictly regulated by the spacing of collagen fibrils (Fratzl et al. 2004), the diameter is supposed to have a maximum value $d_{\text{max}} = 1/5D$ while the length has a maximum value $l_{\text{max}} = 2D$ which is determined by the closed space. The termination of the growth is independently regulated in the two directions; each crystal terminates the growth in the corresponding side of the transverse or the longitudinal direction where it contacts with another crystal or the border of the closed space. These processes continue until the total volume of crystals occupies the specified percentage $P$ in the closed space. The average volumetric percentage of mineral crystals in bone tissue is species-dependent and between 30 and 55% (Fratzl et al. 2004), and we therefore analyzed the percentage density of crystals from 0 to 70%. The structure, completed in the cylindrical closed space, consists of fine cylindrical crystals with various sizes. To evaluate the bending stiffness of this structure, we regarded it as a beam bundled by many fine cylindrical rods with various lengths and radii.

Defining the neutral axis in the cross section, we calculated the bending stiffness of this structure based on Timoshenko’s beam theory including rotational inertia and shear deformation (Weaver et al. 1990). Denoting the flexural displacement by $w$ and the rotation of the cross-sectional area by $\psi$, the equations of motion for the beam subjected to a concentrated force $f(t)$ at $x = l_1$ are expressed as follows

$$\frac{\partial}{\partial z} \left( E I \frac{\partial w}{\partial z} \right) - k' G \left( \frac{\partial \omega}{\partial z} + \psi \right) - \rho A \frac{\partial^2 w}{\partial t^2} = 0$$

$$\frac{\partial}{\partial z} \left( k' G A \left( \frac{\partial \omega}{\partial z} + \psi \right) \right) = -\rho A \frac{\partial^2 \omega}{\partial t^2} + f(t) \delta(z - l_1) = 0$$

where $E$ and $G$ are Young’s modulus and shear modulus respectively. $A$ is a cross-sectional area and $I$ is a secondary moment of inertia. $k'$ denotes the shear coefficient in the Timoshenko beam determined by the cross-sectional shape, and we determined the value by the equation which Cowper proposed for the circular section (Cowper 1966). $\delta(z)$ is the Dirac delta function to express the concentrated force. Assuming that the flexural displacements and the rotations of all the nuclei existing in the cross section are equal so that their values can be represented by $w$ and $\psi$, then the cross-sectional area $A$ and the secondary moment of inertia $I$ are calculated as follows

$$A = \sum_{n=1}^{N_N} \frac{\pi d_n^2}{4}$$

$$I = \sum_{n=1}^{N_N} \frac{\pi d_n^2}{4} \left( \frac{d_n^2}{16} + \frac{r_n^2}{4} \right)$$

where $NN$ is the number of the nuclei existing in the cross section and $r_n$ denotes the distance between the center of each nucleus and the neutral axis of the cross section. To calculate the

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deformation to the concentrated force, we used the finite element method introducing the beam element including rotational inertia and shear deformation, and constructed the stiff matrix based on Timoshenko’s beam theory (Petyt 1998). Obtaining the bending rigidity equivalently calculated by the distribution of cylinders in the cross section for each finite element, we analyzed the deformation under simply supported ends subjected to the concentrated force at the center.

Statistical analysis
All data were expressed as mean±s.d. In the examinations using rats, single comparison was made using an unpaired two-tailed Student’s t-test. \( P<0.05 \) were considered statistically significant.

Results
Marked decrease in bone osteocalcin content by warfarin
Fifty-six rats all successfully received the treatments. Long-term warfarin administration markedly decreased osteocalcin content in humeral mid-shaft cortical bones of 6-month-old rats; the osteocalcin levels in warfarin-administered rats were only 14.8±3.2% of those in vehicle-administered rats (\( n=28 \) each group, \( P<0.001 \)). This confirmed that warfarin effectively inhibited carboxylation of osteocalcin and hence restricted its incorporation into bone. Among these warfarin-administered rats, five showed slight and temporary bleeding from the nose and/or in the eyes, but no other evidence of bleeding was found during the follow-up and after the kill.

Impairment of bone material hardness by warfarin
To test our first hypothesis, we examined the material properties in humeral mid-shaft cortical bones of 6-month-old rats. Consistent with the effect of warfarin on lumbar or hip areal BMD in humans (Caraballo et al. 1999a), peripheral quantitative computed tomography analysis showed that long-term warfarin administration as well as vertical jumping exercise did not change BMD in these bones; BMDs in vehicle- and warfarin-administered rats with no exercise were 1252±26 and 1254±20 mg/cm\(^3\) respectively and BMDs in these rats with vertical jumping exercise were 1246±37 and 1246±20 mg/cm\(^3\) respectively (\( n=14 \) each group). Interestingly, however, analysis of these bones using nanoindentation in a transverse direction revealed that long-term warfarin administration significantly decreased bone material hardness (−9.9±5.0%; Fig. 1A) but did not influence its elastic modulus (0.1±1.6%). In nanoindentation analysis, the hardness was measured during loading (forward), indicating that warfarin would impair the mechanical properties of tiny mineral crystals. In contrast, calculation of the elastic modulus was made during unloading (backward), suggesting that warfarin could not influence the mechanical properties of collagen fibrils.

Decrease in bone mineral size by warfarin
As mineral size in the cortical bone of 6-month-old osteocalcin-deficient mice was smaller than that in age-matched wild-type mice (Boskey et al. 1998), we evaluated mineral size in humeral mid-shaft cortical bones of 6-month-old rats. First, analysis using a scanning acoustic microscope showed that long-term warfarin administration significantly decreased (−4.2±3.6%) bone leaky surface acoustic wave velocity in a longitudinal direction (Fig. 1B). This suggested that warfarin induced structural modification of minerals because BMD in these bones was not changed by the warfarin. In order to strengthen this possibility, we further analyzed the percent area of a 1075 cm\(^{-1}\) sub-band in the \( v_1 \), \( v_3 \) phosphate region of hydroxyapatite spectra, which is positively related to mineral size (Boskey et al. 1998), using Fourier transform infrared microspectroscopy on a longitudinal plane. Consistent with the finding in cortical bone of 6-month-old osteocalcin-deficient mice (Boskey et al. 1998), the percent area of a 1075 cm\(^{-1}\) sub-band was significantly smaller (−10.5±8.4%) in warfarin-administered rats than that in vehicle-administered rats (Fig. 1C). The results, obtained by these two different methods, collectively indicated that long-term warfarin administration diminished mineral size in cortical bone.

Inverse relation between nucleation rate of mineral crystals and their rigidity in computer simulation
As long-term warfarin administration markedly decreased bone osteocalcin levels and since we found that the effects of warfarin involved modification of bone minerals, we sought evidence for a common mechanism of action. Surprisingly, in perfect agreement with the above results in rat cortical bone, our computer-simulation model showed that a delay of mineral crystal nucleation induced by osteocalcin (Hunter et al. 1996) decreased mineral formation rate, increased mean and distribution of mineral sizes, and strengthened mineral rigidity (Fig. 2A–E). In addition, our model showed that mineral crystal growth speed had the opposite effects when compared with mineral crystal nucleation rate (Table 1), which strongly supported the validity of this model because osteopontin, another major non-collagenous protein in bone, mainly inhibits crystal growth without delaying crystal nucleation in mineral formation de novo (Hunter et al. 1996) and mice lacking osteopontin have larger mineral size (Boskey et al. 2002) and stiffer bone tissue (Duvall et al. 2007).

Compensatory adaptation of warfarin-administered bone structure to mechanical stimuli
To test our second hypothesis, we examined the effects of long-term warfarin administration on intrinsic material
properties, morphological parameters, and biomechanical properties in tibial mid-shaft cortical bones of 6-month-old rats with or without vertical jumping exercise. In the present study, warfarin tended to decrease tibial length but did not significantly affect either body weight or tibial length (Table 2), indicating that tibiae in vehicle- or warfarin-administered rats were likely to experience similar mechanical circumstances. In rats with vertical jumping exercise, although long-term warfarin administration significantly increased BMD (+1.3 ± 1.7%), it impaired intrinsic material properties (Table 3); ultimate stress was significantly decreased (−6.2 ± 4.3%). Consistent with our hypothesis, however, we found that long-term warfarin administration induced significant increases in cross-sectional total (+6.2 ± 4.7%) and cortical (+5.8 ± 3.7%) bone areas and tendencies of increases in cross-sectional marrow area (+8.2 ± 13.1%) and cross-sectional moment of inertia (+7.4 ± 8.0%), and did not change biomechanical properties such as ultimate load, stiffness, and work to failure (Table 3). The tibia is a weight-bearing bone independent of vertical jumping exercise and similar changes were observed to a lesser extent in rats with no exercise; however, long-term warfarin administration tended to decrease (−9.4 ± 13.2%) work to failure (Table 3). In warfarin-administered rats, vertical jumping exercise-induced increases in cross-sectional total (+13.5 ± 5.0%, \( P=0.030 \)) and bone (+15.4 ± 4.0%, \( P=0.003 \)), but not marrow (+4.9 ± 12.7%) areas, were significantly higher when compared with those (+9.4 ± 4.2%, +10.5 ± 3.9% and +4.1 ± 8.3% respectively) in vehicle-administered rats. These results indicated that mechanical stimuli countered the impaired material properties of cortical bone, induced by warfarin, through increases in its morphological parameters.

**Discussion**

In the present study, we found that long-term warfarin administration did not change BMD, but markedly decreased osteocalcin content, diminished mineral size, and impaired material hardness in humeral cortical bone of 6-month-old rats. In agreement with these results, based on the findings that osteocalcin mainly delays crystal nucleation without inhibiting crystal growth in mineral formation de novo (Hunter et al. 1996) and the rigidity of bone tissue is produced by tiny mineral crystals (Fratzl et al. 2004), our computer-simulation model suggested that osteocalcin-induced delay of mineral crystal nucleation decreased mineral formation rate, increased mean and distribution of mineral sizes, and strengthened mineral rigidity. In tibial cortical bone of 6-month-old rats, we also found that long-term warfarin administration decreased material ultimate stress but, under vertical jumping exercise, completely maintained biomechanical properties including work to failure through structural expansion. Notably, although warfarin affects not only osteocalcin but also other Gla proteins, these findings are clearly
Figure 2  Effects of nucleation rate of crystals on their size and structural rigidity in conceptual computer-simulation model of mineral crystal formation. (A) Appearance of crystals in a condition of \( n = 100, \; T = 100 \) steps, \( V = 1 \times 10^{-5} \) D/step, and \( P = 60\% \). \( n \), number of crystals which randomly and periodically arise within a closed circular cylindrical space of the diameter \( D (\leq 1 \) μm) and the length \( L = 2D \). \( T \), periodical time; \( V \), growth velocity of crystals; and \( P \), percentage density of crystals in the space. (B) Relationship between progress time and percentage density of crystals (\( T = 100 \) steps, \( V = 1 \times 10^{-5} \) D/step) in conditions of \( n = 50 \) (left), 100 (middle), and 200 (right). (C) Diameter and length of crystals (\( T = 100 \) steps, \( V = 1 \times 10^{-5} \) D/step, and \( P = 60\% \)) in conditions of \( n = 50 \) (left), 100 (middle), and 200 (right). (D) Relative mean and distribution of crystal diameters and lengths in the same conditions of (C). Mean ± s.d. (\( n = 5 \) each group). (E) Relative bending rigidity of crystals in the same conditions of (C). Mean ± s.d. (\( n = 5 \) each group).
consistent with our two hypotheses (Sugiyama et al. 2002a) and cortical bone features of 6-month-old osteocalcin-deficient mice such as a decrease in mineral size (Boskey et al. 1998) and increases in bone thickness and marrow area without changing bone stiffness (Ducy et al. 1996). Indeed, bone strain generated by mechanical loading affects bone structure (Ehrlich & Lanyon 2002); impairment of bone material quality increases bone strain from mechanical loading which could expand cortical bone structure through promotion of periosteal bone formation and endosteal bone resorption.

We used s.c. injection of warfarin at a dose of 0·35 mg/kg per day for 3 alternate days/week (1·05 mg/kg per week) in the present study. Our results in tibial mid-shaft cortical bones of warfarin-administered rats without vertical jumping exercise seem to be similar to a recent finding that s.c. injection of warfarin at a daily dose of 0·25 mg/kg per day (1·75 mg/kg per week), which resulted in the blood coagulation status within the therapeutic range, reduced intrinsic material, and biomechanical properties in femoral mid-shaft cortical bones of approximately 6-months old rats (Simon et al. 2002). Marked decrease in bone osteocalcin content but no apparent skeletal changes in rats treated with high-dose warfarin plus vitamin K1 (Price & Williamson 1981) would be also compatible with our findings, because vitamin K1 can be endogenously converted to menaquinone-4, a vitamin K2 with four isoprenoid residues (Davidson et al. 1998, Ronden et al. 1998), and menaquinone-4 has been recently suggested to improve bone material quality independently of osteocalcin (Ichikawa et al. 2006, Sugiyama 2007). In contrast, however, warfarin monotherapy effectively decreased osteocalcin content in bone but did not alter skeletal status in young adult rats (Haffa et al. 2000). Since no changes were detected in mineral properties at 4 weeks of age (Boskey et al. 1998) and bone phenotype became noticeable after 6 months of age (Ducy et al. 1996) in osteocalcin-deficient mice, the effect of osteocalcin on bone material quality appears to be hidden in relatively high-turnover bones. This possibility could be supported by the finding in 6-month-old osteocalcin-deficient mice after 1 month from ovariectomy that mineral size was decreased in the center and endostium of cortical bone, but not in the periosteum of cortical bone or trabecular bone when compared with their wild-type control mice (Boskey et al. 1998), because mouse bones are modeled but not remodeled and thus newly formed minerals under the ovariectomized-induced high bone turnover in these mice would be included in the periosteum of cortical bone and trabecular bone, but not the center and endosteum of cortical bone.

Warfarin has been suggested to increase fracture risk by impairing bone quality (Eastell 2006), but does not change bone turnover, which strongly affects bone quality, in humans (Knapen et al. 2000) as well as in rats (Amizuka et al. 2005) and rhesus monkeys (Binkley et al. 2007). Our findings suggest that long-term use of warfarin weakens the rib and vertebra by impairing cortical bone material quality due to a marked decrease in osteocalcin content.

Table 1 Effects of growth speed of crystals on their size and structural rigidity in conceptual computer-simulation model of mineral crystal formation (n=100, T=100 steps, and P=60%). Mean±s.d. (n=5 each group)

<table>
<thead>
<tr>
<th>Progress time (steps)</th>
<th>Diameter of crystals (1×10^-2D)</th>
<th>Length of crystals (1×10^-2D)</th>
</tr>
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<tbody>
<tr>
<td>Mean</td>
<td>2·578±0·004</td>
<td>3·628±0·016</td>
</tr>
<tr>
<td>Distribution</td>
<td>1·644±0·005</td>
<td>2·580±0·012</td>
</tr>
<tr>
<td>Relative bending rigidity of crystals</td>
<td>0·350±0·037</td>
<td>1·000±0·114</td>
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</table>

Table 2 Body weight and tibial length of long-term vehicle- or warfarin-administered rats with or without vertical jumping exercise. Mean±s.d. (n=14 each group)

<table>
<thead>
<tr>
<th></th>
<th>No exercise</th>
<th>Vertical jumping exercise</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>Warfarin</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 week old</td>
<td>151±6</td>
<td>152±6</td>
</tr>
<tr>
<td>16 week old</td>
<td>156±6</td>
<td>160±6</td>
</tr>
<tr>
<td>20 week old</td>
<td>164±6</td>
<td>166±6</td>
</tr>
<tr>
<td>24–27 weeks old</td>
<td>176±7</td>
<td>176±6</td>
</tr>
<tr>
<td>Tibial length (mm)</td>
<td>37·0±0·5</td>
<td>36·5±0·5</td>
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</table>

No significant differences between vehicle and warfarin.

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In contrast, because the degree of bone strain from mechanical loading in ambulant people is much higher at the hip than at the rib or vertebra, long-term warfarin therapy could not reduce hip strength through compensatory adaptation of cortical bone structure to higher mechanical stimuli. Indeed, we previously found an inverse correlation between the baseline value of urinary carboxyglutamate, a possible parameter of osteocalcin carboxylation, and the percentage change of whole bone areal BMD after 6-month vertical jumping exercise in healthy premenopausal women (Sugiyama et al. 2002b).

In contrast, however, low dietary intakes of vitamin K₁ (Booth et al. 2000) as well as high levels of non-carboxylated or low levels of carboxylated osteocalcin in blood (Szulc et al. 1993, 1996, Vergnaud et al. 1997, Luukinen et al. 2000, WHO Scientific Group 2003) are associated with increased risk of hip fracture in elderly people. These contradictory findings can be explained by the different degrees of bone osteocalcin content; in addition to higher mechanical stimuli, marked decrease in osteocalcin content could be required to induce enough bone strain for the expansion of cortical bone structure. This possibility is supported by a recent finding that only a lower accumulated dose of vitamin K antagonists was related to increased risk of fracture (Rejnmark et al. 2007), and no effect of vitamin K₁ on fracture risk in perimenopausal and early postmenopausal women (Rejnmark et al. 2006) can be explained by the suggestion mentioned above that the effect of osteocalcin on bone material quality is reduced in high-turnover bones. Similarly, higher volumetric BMD in elderly men when compared with that in elderly women (Russo et al. 2006) could explain why long-term use of warfarin resulted in the increased risk of fractures in men but not in women and this gender difference arose primarily from hip fracture (Gage et al. 2006), because higher volumetric BMD induces lower bone strain from mechanical loading.

Bone phenotype in mice lacking apolipoprotein E is similar to that in osteocalcin-deficient mice (Schilling et al. 2005) and thus our findings will provide a new insight into the relationship between apolipoprotein E4 allele and fracture risk. Apolipoprotein E4 allele is associated with the low availability of vitamin K to bone (Newman et al. 2002), but the relation between this allele and fracture risk remains inconsistent (Cauley et al. 1999, Schoofs et al. 2004). Possible explanations include differences in age, gender, body weight, physical activity, and vitamin K intakes; different degrees of poor bone material quality with or without compensatory adaptation of bone structure to mechanical stimuli could influence the fracture risk. Apart from warfarin and vitamin K, various factors appear to affect cortical bone material quality through osteocalcin (Sugiyama & Kawai 2004, Sugiyama et al. 2005). For example, type 2 diabetic patients have an increased fracture risk independently of areal BMD (Schwartz et al. 2001, Strotmeyer et al. 2004) and their circulating osteocalcin levels are low (Okazaki et al. 1997). Consistent with our results, mid-shaft cortical bones of type 2 diabetic rats showed expansion of periosteal and endosteal circumferences (Ahmad et al. 2005) and vitamin K treatment improved bone fragility by modifying minerals in these rats (Wada et al. 2000).

In conclusion, our findings are strongly supported by several lines of evidence and thus would provide a new significant insight into the mechanisms by which warfarin affects the skeleton. Although further studies are essential, these findings can explain the controversial evidence concerning the relationship between long-term warfarin use and fracture risk in older patients, suggesting that the present

### Table 3 Bone mineral density, intrinsic material properties, morphological parameters, and biomechanical properties in tibial mid-shaft cortical bones of long-term vehicle- or warfarin-administered 6-month-old rats with or without vertical jumping exercise. Mean±s.d. (n = 13–14 each group)

<table>
<thead>
<tr>
<th></th>
<th>No exercise</th>
<th>Warfarin</th>
<th>Vertical jumping exercise</th>
<th>Warfarin</th>
</tr>
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<tbody>
<tr>
<td><strong>Bone mineral density</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mg/cm³)</td>
<td>1237±24</td>
<td>1236±12</td>
<td>1255±21</td>
<td>1272±21*</td>
</tr>
<tr>
<td><strong>Intrinsic material properties</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ultimate stress (MPa)</td>
<td>319±20</td>
<td>303±20*</td>
<td>322±15</td>
<td>302±14§</td>
</tr>
<tr>
<td>Young's modulus (GPa)</td>
<td>6.33±1.03</td>
<td>6.16±0.74</td>
<td>5.76±1.07</td>
<td>5.27±0.77</td>
</tr>
<tr>
<td>Toughness (MJ/m³)</td>
<td>13.3±2.7</td>
<td>11.7±1.9</td>
<td>13.4±2.1</td>
<td>12.4±1.7</td>
</tr>
<tr>
<td><strong>Morphological parameters</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cross-sectional total area (mm²)</td>
<td>3.20±0.14</td>
<td>3.28±0.14</td>
<td>3.51±0.14</td>
<td>3.72±0.17*</td>
</tr>
<tr>
<td>Cross-sectional bone area (mm²)</td>
<td>2.65±0.14</td>
<td>2.68±0.12</td>
<td>2.92±0.10</td>
<td>3.09±0.11*</td>
</tr>
<tr>
<td>Cross-sectional marrow area (mm²)</td>
<td>0.560±0.046</td>
<td>0.601±0.042†</td>
<td>0.583±0.046</td>
<td>0.631±0.076</td>
</tr>
<tr>
<td>Cross-sectional moment of inertia (mm⁴)</td>
<td>0.660±0.063</td>
<td>0.688±0.064</td>
<td>0.800±0.090</td>
<td>0.859±0.064</td>
</tr>
<tr>
<td><strong>Biomechanical properties</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ultimate load (N)</td>
<td>77.8±5.2</td>
<td>76.7±6.6</td>
<td>89.0±7.2</td>
<td>89.7±6.6</td>
</tr>
<tr>
<td>Stiffness (N/mm)</td>
<td>200±25</td>
<td>202±21</td>
<td>218±32</td>
<td>216±28</td>
</tr>
<tr>
<td>Work to failure (N×mm)</td>
<td>25.0±5.6</td>
<td>22.6±3.3</td>
<td>27.0±5.0</td>
<td>26.7±4.1</td>
</tr>
</tbody>
</table>

*P=0.039; †P=0.019 versus vehicle under no exercise; ‡P=0.046; §P=0.001; ††P<0.001 versus vehicle under vertical jumping exercise.
concept could be translated into the clinical situation in humans.

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